AN INVESTIGATION INTO THE ROLE OF TLR3 ACTIVATION ON HIPPOCAMPAL FUNCTION

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AUTHOR'S DECLARATION

All the work in this thesis was carried out solely by the author. This thesis has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signature:

Date:

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ABSTRACT

Toll like receptors (TLRs) belong to a family of pattern recognition receptors that recognise broadly shared molecules found on pathogens referred to as pathogen associated molecular patterns (PAMPs). TLRs are well known for their involvement in innate immunity however despite their presence in the CNS, our knowledge of their function in the CNS is limited. Therefore in the present study, we investigated the cellular localisation of TLR3 and the consequence of its activation on synaptic activity.

Primary hippocampal cultures using P1-2 rats were prepared and used experimentally between 10-14DIV. Standard whole cell patch clamp electrophysiology recordings in voltage and current clamp were used to monitor ion channel function and synaptic activity respectively.

Experiments revealed TLR3 expression in neurons, astrocytes and oligodendrocytes within the cultures. Synaptically driven spontaneous action potential (AP) firing was significantly reduced by short-term application (5min) of the TLR3 specific activator, poly I:C (25µg/ml and 200µg/ml). Furthermore long-term poly I:C application (1hr) showed a dramatic reduction in AP firing (1µg/ml and 25µg/ml). Investigations were carried out to determine the mechanisms underlying these effects. Short-term application of poly I:C (200µg/ml) had no effect on the frequency and amplitude of miniature excitatory postsynaptic currents (mEPSCs). However, a significant reduction on sodium current was observed. In contrast, long-term application of poly I:C (25µg/ml) resulted in a significant reduction in mEPSC amplitude, frequency and peak sodium and potassium current. Furthermore long-term application

of poly I:C (25µg/ml) resulted in a significant reduction of surface AMPAR expression. These underlying effects of TLR3 activation require the activation of the TRIF pathway as shown by the mutant version of TLR3 blocking the effect on sodium current.

In summary, these data imply that TLR3 activation modulates hippocampal synaptic activity through multiple mechanisms. This data might provide further insight into how this contributes to virally-mediated behavioural changes.

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AMPARs	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
APCs	Antigen presenting cells
sAP	Spontaneous action potential
AraC	Cytosine β-D-arabinofuranoside
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CamKII	Ca_2^+/calmodulin-dependent protein kinase II
CNS	Central nervous system
CSPα	Cysteine string protein alpha
DAMPs	Damage-associated molecular pattern molecules
DIV	Days *in vitro*
DL-AP5	DL-2-Amino-5-phosphonovaleric acid
DRG	Dorsal root ganglion
ECDs	Extracellular ligand binding domain/ectodomain
EDTA	Ethylene-di-amine tetra-acetic acid
EGTA	Ethylene glycol tetra-acetic acid

EPSCs	Excitatory postsynaptic currents
E-LTP	Early long term potentiation
mEPSC	Miniature excitatory post synaptic current
FBS	Foetal bovine serum
GABA	Gamma-Aminobutyric acid
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GPCR	G-protein coupled receptors
GTP	Guanosine-5'-triphosphate
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hsc70	Constitutive heat shock protein 70
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL	Interleukin
iPSC	Induced pluripotent stem cells
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory transcription factors
ISRE	Interferon sensitive response element
K^+	Potassium
KCl	Potassium chloride
$KMeSO_2$	Potassium methyl sulphate

KO	Knock-out
LASAF	Leica image capturing software program
LPS	Lipopolysaccharide
LRRs	Leucine rich repeats
LTD	Long term depression
LTP	Long term potentiation
MAP-2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential medium
$MgSO_4$	Magnesium sulphate
MS	Multiple sclerosis
Munc-18	Mammalian uncoordinated -18 protein
MyD88	Myeloid differentiation primary response gene 88
Na^+	Sodium
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
NaH_2PO_4	Sodium dihydrogen phosphate
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NMDARs	N-methyl-D-aspartate receptors
NO	Nitric oxide
NOS-2	Nitric oxide synthase 2
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PKC	Protein kinase C
PKMʒ	Protein kinase m zeta
PLC	Phospholipase C
PNS	Peripheral nervous system
Poly A:U	Polyadenylic–polyuridylic acid
Poly I:C	Polyinosinic:polycytidylic acid
PRRs	Pathogen recognition receptors
SEP-GluA1	Super ecliptic pHluorin-GluA1
SGT	Glutamate and threonine rich protein
SNARE	Soluble NSF attachment receptor
VGLUTS	Vesicular glutamate transporters
TAB	TAK1 binding protein
TAK1	TGF-beta activated kinase 1
TIR	Toll/IL-1R resistance
TLRs	Toll like receptors

TMEV	Theiler’s murine encephalomyelitis virus
TNF-α	Tumour necrosis factor alpha
TRAF6	TNF Receptor-Associated Factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
TTX	Tetrodotoxin
WT	Wildtype

1. INTRODUCTION

1.1. General introduction to the central nervous system

The central nervous system (CNS) and peripheral nervous system (PNS) are the two main parts that make up the highly organised and intricate nervous system of multicellular organisms (Broughton and Partridge, 2009). The CNS itself has seven parts consisting of the cerebellum, diencephalon and cerebral hemisphere which compose the brain protected by the skull. The remaining parts make up the brain stem which is composed of the midbrain, pons and the medulla oblongata and the spinal cord which is protected by vertebrae. Each structure of the CNS has its own role but is critical for the overall purpose of the CNS which is to receive and process all the information sent to it from different parts of the body (Kandel *et al.*, 2000; Brodal, 2004).

Historically, the CNS was considered to be immune privileged with its activities mutually exclusive to that of the immune system. The blood brain barrier (BBB) is essential for the everyday homeostatic conditions of the CNS which is strictly regulated. It is made up of endothelial cells connected by gap junctions to create a barrier which is highly permeable (Anderson *et al.*, 2011). It only allows the passage of certain substances into the brain such as oxygen and carbon dioxide, nutrients such as glucose but more importantly, prevents the entry of pathogens that have the ability to cause damage by destroying the vulnerable tissue of the brain (Ballabh *et al.*, 2004). Furthermore, certain cells of the CNS are in constant surveillance of their surroundings with the role of removing

debris and providing defence consistently against potentially harmful threats such as pathogens. When the threat is recognised, the immune system is activated allowing the passage of other activated immune cells through the BBB to occur to aid the CNS cells in the removal of the threat (Yang *et al.*, 2010; Arima *et al*., 2013).

1.2. Cell types of the CNS

1.2.1. Neurons

Neurons are the basic information processing structures of the CNS that process information received by a stimulus and transmit the information to other neurons. The way in which the information is encoded is through electrical impulses known as action potentials that carry the information. Therefore, a complex structure is required for neurons to carry out this function and they do this through billions of neurons forming a network throughout the CNS allowing information to flow and be acted upon (Gross, 2008). Although there are a variety of different neurons performing specific functions, they all share a general structure. The general structure of a neuron (Fig 1.1) consists of a cell body, sometimes known as the soma, controlling the metabolic and manufacturing state of a neuron, from the cell body emanates dendrites that receive stimuli from other neurons and an axon where action potentials are passed on as an outgoing signal to other neurons after being processed (Arbib *et al.*, 1998).

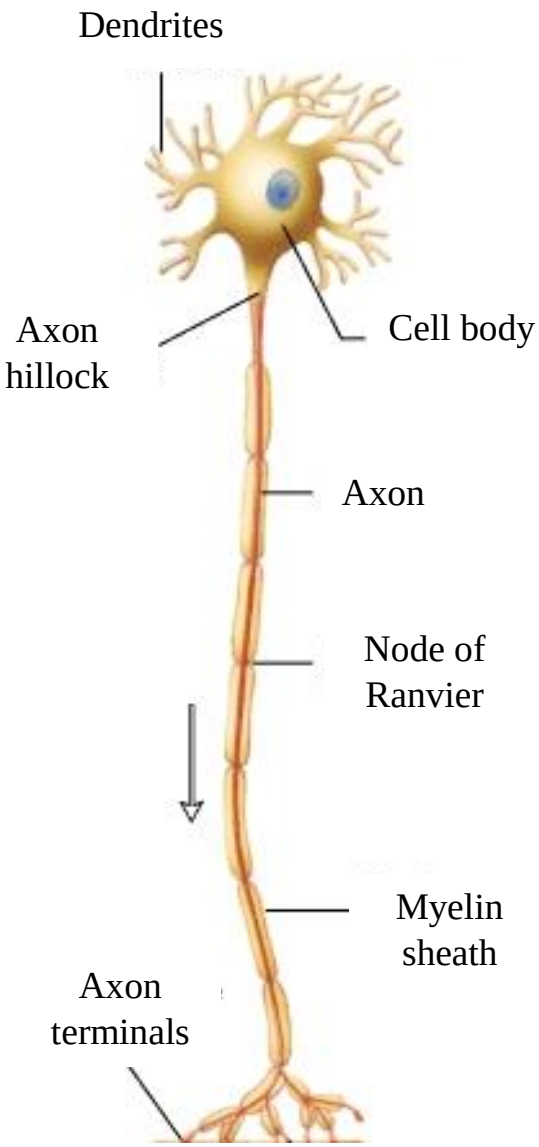


Figure 1.1: The typical structure of a neuron (adapted from Lodish *et al.*, 2000).

Between axon terminals and dendrites, synapses occur. These are the connections between a presynaptic terminal and a postsynaptic neuron allowing neurons to communicate by means of synaptic transmission, either chemically or electrically. A concept has been proposed known as the tripartite synapse (Fig 1.2) where it refers to the presynaptic and post

synaptic elements along with the close association of astrocytes surrounding them. The three components of the tripartite synapse function in the processing of synaptic integration at the chemical synapse (Perea *et al.*, 2009; Tanaka *et al.*, 2010).

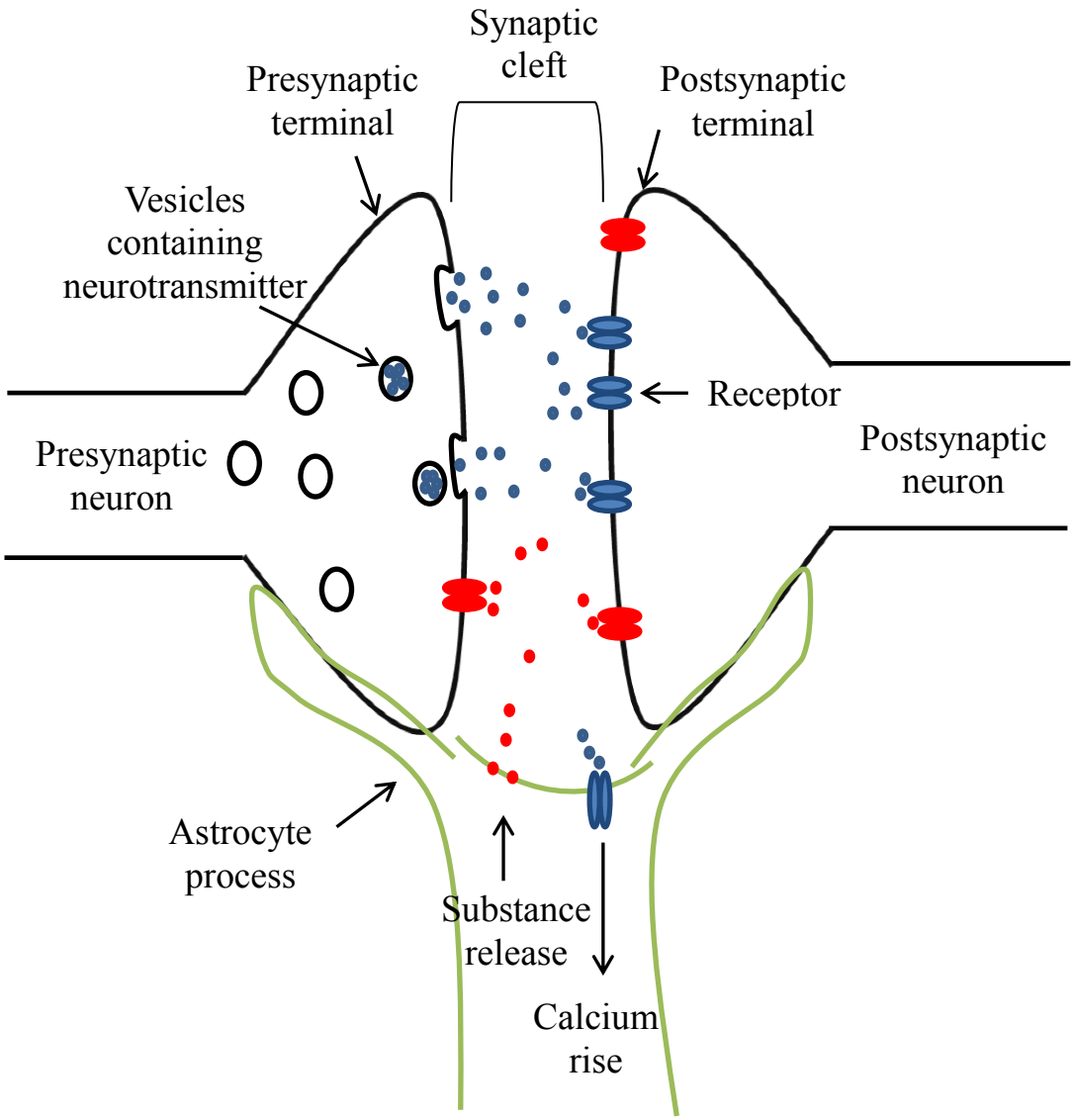


Figure 1.2: The tripartite synapse.

1.2.2. Astrocytes

Astrocytes are the most abundant macroglial cell type within the CNS with diverse functions and morphology (Young *et al*., 2010). No region of the brain is devoid of astrocytes and they have been shown to span the CNS contiguously and in a well-structured manner and are linked together through gap junctions (Sofroniew and Vinters, 2010). The origin of astrocytes has not been fully derived however it has been speculated that they are generated from a restricted region of the neural tube (Chaboub and Deneen, 2012). Astrocytes can either arise from immature cells from the dorso-ventral subventricular region of the brain or from the ventricular zone and form late in embryogenesis (Trotter *et al.,* 2010). Classically, astrocytes do not have any electrical excitability and even though they express sodium and potassium voltage-gated ion channels, they maintain a relatively stable membrane potential (Orkand *et al*., 1966). However, it is now known that astrocytes are excitable cells in relation to levels of intracellular calcium (Cornell-Bell *et al*., 1990; Araque and Navarette, 2010; Parpura and Verkhratsky, 2012).

There are several types of astrocytes including fibrous and protoplasmic. Fibrous astrocytes are located in the white matter whereas protoplasmic astrocytes, which are more prevalent and connect with synapses, populate the grey matter of the brain (Molofsky *et al.*, 2012). They are known to take part in the formation of the BBB and interact with cells of the immune system where if activated, can play a role as innate immune cells themselves leading to an inflammatory state in the CNS. This state leads to an increase in BBB permeability permitting the modulation of

monocyte and lymphocyte migration along with inflammatory mediator release (Weber *et al.*, 1994). These mediators include TNF-α, IL-1β and IL-12 that lead to the amplification of the inflammatory state in the immediate environment and can result in damage to the tissue increasing the possibility of neurodegeneration (Farina *et al.*, 2007; Trudler *et al.*, 2010). However, astrocytes not only have an immune role but contribute to the homeostasis of the CNS by providing structuring of the brain, forming a glial scar when injury occurs to neurons and replacing those that cannot regenerate, regulating ion concentrations, along with a supporting role to neurons trophically, supplying nutrients and glycogen during periods of high energy consumption, promoting their survival and their differentiation (Korn *et al.*, 2007). In addition, they modulate neuronal excitability and synaptic transmission leading to the proposal of the tripartite synapse, as mentioned previously. It was suggested this role was through calcium elevations and gliotransmitter release such as glutamate (Araque *et al.*, 1998). Subsequently, studies proved the theory by showing that these act on NMDA receptors on neighbouring neurons causing depolarisation therefore modulating the excitability and strength of neurons (Verkhratsy and Kirchoff, 2007; Sild and Van Horn, 2013; Lalo *et al*., 2014).

1.2.3. Microglia

Microglia are known to be the innate immune cells of the CNS constituting 10% of cells in the brain. They are the resident macrophages and are therefore the initial first line of defence in the CNS (Ross, 2010). They originate from hematopoietic stem cells of the monocyte and

macrophage lineage where they differentiate into monocytes and travel to the brain. Within the CNS, they are derived from the mesoderm where evidence has shown them to migrate into the CNS during embryogenesis where they mature (Derecki *et al.,* 2013). Microglia are adaptable in the sense that depending on the region of the brain they are located in and the role they play there, they can change their structure to suit the requirements needed (Graeber *et al.,* 1988).

Historically they were considered to be in a resting state under normal physiological conditions of the CNS, however more recent studies have proven this statement to be wrong. These cells are always scavenging and the reason for this is to contribute to the homeostasis of the brain (Dibaj *et al.,* 2011). To do this, microglia cells endocytose any useful nutrients in their local surroundings whilst constantly being on alert for potential threats. In addition, they remove any cells that have died through apoptosis in addition to debris by a process called phagocytosis, to prevent inflammation (Napoli and Neumann, 2009). A study showed that microglia survey the local area through ramified processes. During *in vivo* microscopy on mice it was revealed that these cells have high motility and the processes which are filopodia like protusions, allow the cells to survey their surroundings for threats through the extension and retraction of these processes (Hanisch and Kettenmann, 2007; Kress *et al.,* 2007; Dibaj *et al.,* 2011).

However under pathological conditions, microglia are involved in the first line of defence and are amongst the first cells to be present at the site of infection and injury. When a threat presents itself, microglia proliferate rapidly as due to the nature of the BBB, it is difficult to

replace microglia and as few antibodies are small enough to pass through, microglia need to act quickly (Cătălin *et al.,* 2013). Microglia become attracted to the site of injury through the release of nucleotides from damaged neurons (Haynes *et al.,* 2006). Microglial cells then release chemokines which further attract cells to the site of damage whereas pro-inflammatory mediators such as (but not limited to) nitric oxide (NO) produced by the enzyme nitric oxide synthase 2 (NOS-2), hydrogen peroxide, reactive oxygen species, TNF-α, IL-12 and IL-6 are involved in pathogen elimination. Subsequently, these cells remove the remains of pathogens through phagocytosis (Koizumi *et al*., 2007). However not only do they eliminate pathogens and infected neurons, they can also cause damage to the healthy neurons that aren't affected which may lead to chronic inflammation (Mehler and Kessler, 1997). Due to this nature, microglial cells are generally described as neurodestructive, however they have the capability to be anti-inflammatory too (Michelucci *et al*., 2009). In addition, microglial cells have the ability to be antigen presenting cells where they phagocytose the antigen of a pathogen and present the antigen to T cells which in turn, causes cytokine release amongst other roles (Wlodarczyk *et al.,* 2014). After the elimination of the pathogen and post-inflammation, microglia play a role in promoting repair and remapping when required through anti-inflammatory secretion and neuronal tissue regrowth (Welser-Alves *et al.,* 2011).

1.2.4. Oligodendrocytes

Oligodendrocytes are critical cells of the CNS as they are responsible for the myelination of neurons and originate from ectodermal cells (Shin *et al.*, 2012). They exist in two developmental stages, immature and mature and are the last cell type of the CNS to be generated during development (Bradl and Lassmann, 2010). The immature oligodendrocytes can proliferate however they do not have the capability to cause myelination whereas mature oligodendrocytes can no longer proliferate but do allow myelination to occur on neurons by creating a sheath of myelin consisting of protein (20%) and lipid (80%), (Keirstead and Blakemore, 1999). This process begins in a few regions of the brain at birth and continues into adulthood (Jung *et al.*, 2010). Myelination occurs at the axonal region allowing fast and efficient conduction of electrical impulses (signals) along neurons. However there is accumulating evidence that myelination is not the only role they perform. An example of a suggested role proposed for oligodendrocytes is through lactate transporters, thereby providing metabolic support to axons and neurons to sustain energy demands (Rinholm *et al.*, 2011).

As previously mentioned, oligodendrocytes are critical cells and if damaged, they can lead to demyelinating diseases such as Multiple Sclerosis (MS) where the major feature is the plaque characterised by the death of oligodendrocytes resulting in a loss of myelin that ensheaths axons. This can result in motor and sensory deficits ultimately leading to blindness and paralysis (Stariha and Kim, 2001; Coelho *et al.*, 2010).

1.3. General introduction to synaptic transmission

1.3.1. Communication between neurons

In general, neurons communicate in a stepwise manner through means of synaptic transmission or neurotransmission as it can also be called (McMahon, 1994; Pereda, 2014). To begin this process, the neuron must receive a stimulus either from the environment or from another neuron resulting in the processing of the information encoded from the stimulus leading to an electrical signal (containing the information) to propagate along the axon at fast speed. The neuron will then convert the electrical signal into a chemical signal allowing the information to be passed onto another neuron (Lee *et al.*, 2010). However, the mechanism as to how the process starts is extremely important. Neurons are unique cells in the manner that they maintain a stable membrane potential of around -60mV to -70mV so that depolarisation of the neuron, which begins the process mentioned, can be controlled and prevent overstimulation. Depolarisation occurs from an altered balance of ions inside the neuron (increase in sodium) and outside the neuron (increase in potassium) therefore the neuron maintains its resting membrane potential through actively transporting sodium out of the neuron and potassium into it (Wladyka and Kunze, 2006; Hu *et al*., 2009).

As previously mentioned, a neuron has to receive a stimulus first and foremost to allow synaptic transmission to occur. The mechanism to how this occurs is known as conduction where the neuron receives a stimulus, interprets it and then has to communicate with its axon before passing

the signal onto another cell (Lodish *et al.*, 2000). The parts of the cell body that receives the stimulus are the dendrites as they contain receptors at synapses and results in the information being processed as an action potential along the neurons axon to the axon terminals. However for an action potential to be generated, it must reach the excitation threshold. This occurs when the cell membrane depolarises past the threshold of excitation as the result of a stimulus (Burke *et al*., 2001; Platkiewicz and Brette, 2010). Once the threshold is reached, voltage-dependent sodium channels in the axon hillock become activated and open allowing influx of sodium resulting in complete depolarisation and an action potential is triggered which then travels down the axon. The sodium channels are open for about 1ms allowing approximately 6000 sodium ions to pass through the membrane before becoming inactive. This stage is known as the sodium refractory period allowing the inside membrane potential to restablish before closing. This primes the channels for further depolarisation (Lodish *et al.,* 2000). Voltage dependent potassium channels open allowing potassium out of the cell and results in hyperpolarisation of the neuron. This enables sodium channels to return to their steady state (Chu and Zhen, 2010). Any extra potassium ions diffuse out of the cell allowing the return of its resting membrane potential (Fig 1.3). To help the neuron membrane potential reach its resting state, active transport of potassium ions and sodium ions by the sodium-potassium pump occurs (one cycle moves 3 sodium ions and 2 potassium ions) also resulting in action potentials travelling in one direction (Cobbett *et al*., 1987; Boeiro *et al*., 2005). When an action potential arrives at the nerve terminal, synaptic transmission follows.

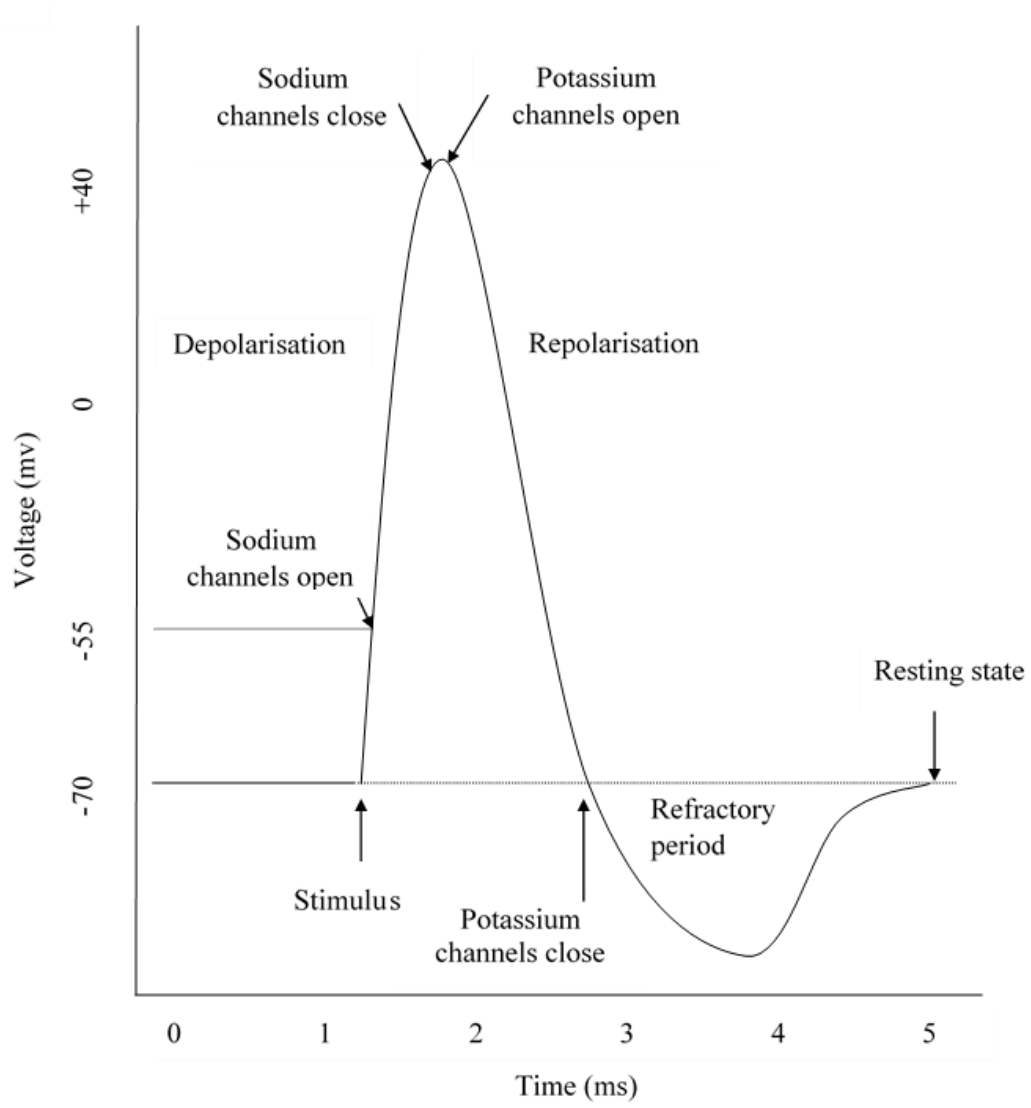


Figure 1.3: The stages propagating the firing of an action potential.

1.3.2. Synaptic transmission

Synaptic transmission is the communication of neurons with each other and effector cells mainly through chemical signals however electrical signals can also occur at the axon terminals where synapses are located. It is found that these two types of synaptic transmission in animals can

co-exist (Szabo *et al.,* 2004). Chemical synaptic transmission is a complex process involving excitatory or inhibitory neurotransmitters that are released from the presynaptic terminal and bind to postsynaptic receptors in a tightly regulated manner signalling to other neurons or non-neuronal cells such as muscles (Evans *et al.,* 2011). On the other hand, electrical synaptic transmission is bi-directional providing a conductive link to send signals between neighbouring neurons, is mainly excitatory and faster than chemical synaptic transmission (Pereda, 2014).

The action potential becomes converted to a chemical signal through neurotransmitter release presynaptically (Daw *et al*., 2009). The neurotransmitter is synthesised in the nerve terminal and then stored in vesicles until required. The presynaptic nerve terminal contains hundreds of vesicles containing neurotransmitter ready for release when an action potential arrives at the axon terminals (Takamori *et al.,* 2006). Two pools of vesicles exist at the presynaptic neuron terminals, which are the readily releasable pool situated close to the membrane and the reserve pool which functions to restore the readily releasable pool when required (Baldelli *et al*., 2007). In response to the generation of an action potential, an increase in cytosolic calcium occurs, opening voltage-gated calcium channels at the nerve terminal leading to an increase in calcium levels, resulting in the triggering of vesicle discharge into the synaptic cleft through calcium binding to its sensor, synaptotagmin (Heuser *et al.,* 1979; Cordeiro *et al.,* 2013). This results in the docking and fusion of vesicles containing neurotransmitters (Figure 1.4) with the membrane through the mechanism of exocytosis (Meunler *et al.,* 2010). Vesicular SNARE protein (soluble NSF attachment receptor) known as synaptobrevin was first identified to be essential for protein fusion

(Haberman *et al*., 2012). Shortly afterwards, the membrane fusion machinery was fully identified with the addition of two further components called SNAP-25 and synatxin-1 which form a complex providing the fuel required for fusion (Blasi *et al.,* 1993). The SNARE complex mediated by an essential SM protein, Munc18-1, which interacts with syntaxin-1 causing fusion-pore opening resulting in complete fusion and merging of the vesicle membrane with the synaptic nerve terminal membrane (Rathore *et al.,* 2010). At the synapse, there are many different SNARE complex conformations therefore to ensure correct conformation, chaperone proteins consisting of a complex made of cysteine string protein α (CSPα), SGT (glutamate and threonine rich protein), Hsc70 and synucleins are required (Sharma *et al.,* 2011; Südhof and Rizo, 2011). All vesicles are released in quanta which is the minimal unit to cause the change in potential of the postsynaptic neuron across the synaptic cleft (Fatt and Katz, 1952).

Following release, the vesicular components are endocytosed and used to recycle new vesicles whilst the neurotransmitter that has been released will be recognised by a receptor on the postsynaptic neuron. This leads to the transmission of the action potential if excitatory such as L-glutamate or if inhibitory such as GABA, will prevent action potential generation. Neurotransmitters will interact with receptors of the postsynaptic cell's membrane causing either the opening or closing of ion channels depending on the nature of the neurotransmitter and the receptor it interacts with (Belousov *et al*., 2001; Hyzinski-García *et al*., 2009). Inactivation of the neurotransmitter must occur to prevent constant stimulation of the postsynaptic neuron and allow another action potential to generate if further neurotransmitters are released (Wu *et al*.,

2006). For example, inactivation of L-glutamate can occur via reuptake by astrocytes converting L-glutamate to L-glutamine or via reuptake into the postsynaptic or presynaptic compartment. The synaptic cleft is a space between both neurons that prevents them directly transmitting action potentials and synapses are intercellular junctions between excitable cells. Any step that is dysfunctional often causes an imbalance in the brain and may potentially lead to conditions such as schizophrenia, dementia and Alzheimer's disease (Lüscher and Isaac, 2009).

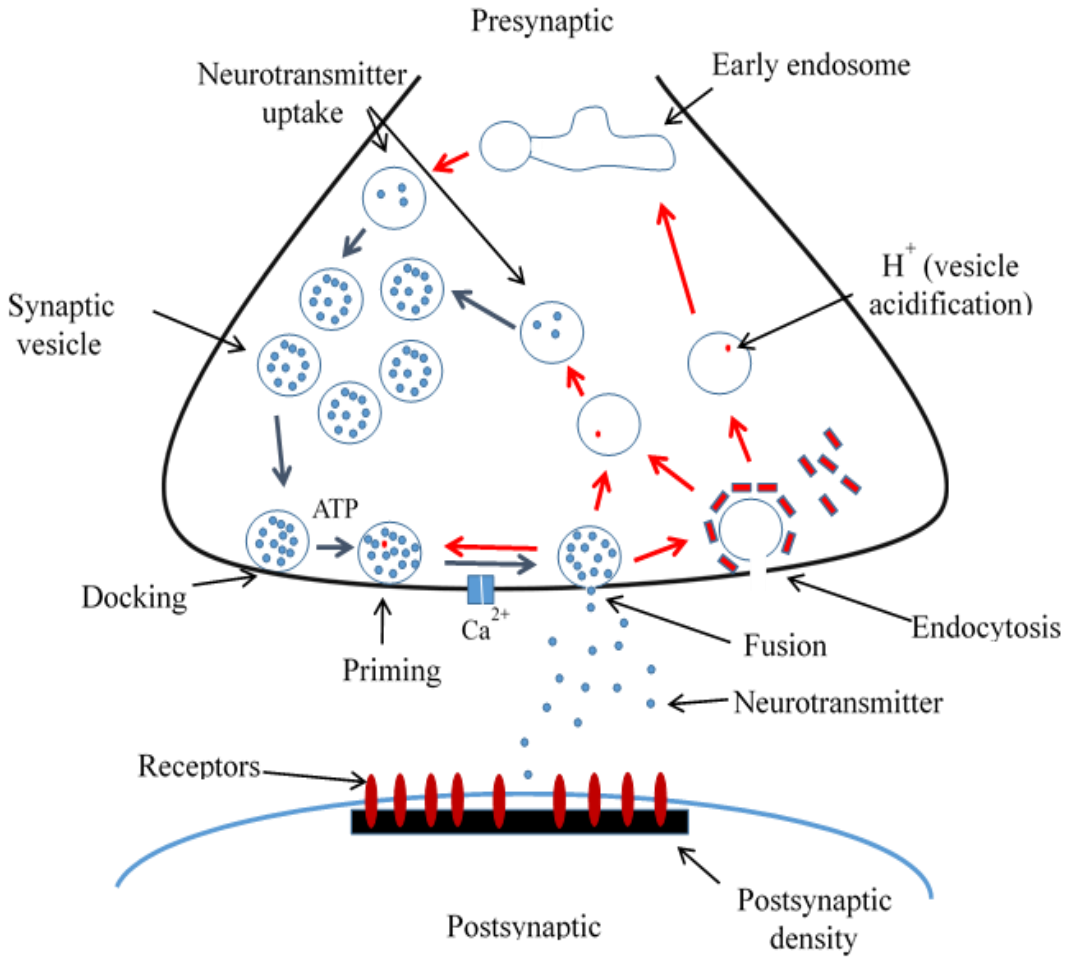


Figure 1.4: The synaptic vesicle cycle including exocytosis (blue arrows) and endocytosis (red arrows).

1.3.3. Glutamate neurotransmission

Excitatory neurotransmission is involved in normal synaptic transmission where the excitatory neurotransmitter, glutamate, is the key player involved (Curtis *et al*., 1959; Erecinska and Silver, 1990). Before glutamate can act, it has to be synthesised and this is primarily done through glucose and α-ketoglutarate. However glutamine released from glial cells can also provide neurons with glutamate after it has been transported to the neuron and does this through an enzyme called glutaminase which converts the glutamine to glutamate (Daikhinn and Yudkoff, 2000; Hyzinski-Garcia *et al*., 2009). After glutamate synthesis, it is stored in vesicles by vesicular glutamate transporters (VGLUTs) until stimulated to be released into the synaptic cleft in a calcium-dependent manner. There are 3 types of these transporters named 1-3 with VGLUT1 being the most abundant in the hippocampus with small amounts of VGLUT3 present also (Shigeri *et al.,* 2004; Ni and Parpura, 2009). Once glutamate has been released into the synaptic cleft, receptors on the postsynaptic neuron will recognise it however it must be removed from the cleft quickly because it will cause excitotoxicity if present in excess (Gras *et al.,* 2006). Therefore to prevent this from happening, glutamate that hasn't bound to the postsynaptic neuron will either be retaken up into the presynaptic neuron or taken up by astrocytes. However, the glutamate bound to the postsynaptic neuron results in an increase in the influx of cations such as sodium and calcium depending on the receptor activated and the efflux of potassium resulting in the depolarisation of the neuron (Van Den Bosch *et al*., 2006; Chu and Zhen, 2010).

The glutamate receptors present on the postsynaptic neuron consist of two classes of which those that act as ion channels are known as ionotropic receptors and those that when activated are linked to intracellular second messengers known as metabotropic receptors. Ionotropic receptors consist of NMDA receptors (NMDARs), AMPA receptors (AMPARs) and kainate receptors (Marmiroli and Cavaletti, 2012).

1.3.4. Ionotropic glutamate receptors

Ionotropic glutamate receptors are transmembrane ligand gated ion channels which allow the passage of ions after recognition of a ligand which in this case is the neurotransmitter, glutamate (Traynelis *et al.*, 2010). They convert the chemical release of neurotransmitter from the presynaptic neuron into an electrical signal at the postsynaptic neuron (Pereda, 2014). As previously mentioned, ionotropic glutamate receptors are composed of three types: NMDARs, AMPARs and kainate receptors.

1.3.4.1. NMDA receptors

NMDARs (N-methyl-D-aspartate receptors) are classically found on the postsynaptic neuron and have the highest affinity for glutamate. They form heteromeric assemblies composed from GluN1, GluN2A, GluN2B, GluN2C, GluN2D, GluN3A and GluN3B subunits. Assembly of the

receptor as a di-heteromer is the minimal requirement for it to be functional *in vitro*, however an NMDA receptor can also form a more complex tri-heteromeric structure both *in vitro* and *in vivo* (Dingledine *et al.*, 1999). NMDARs have distinct features including being ligand-gated and voltage-dependent however for its activation, it requires both glutamate and either glycine or D-serine as a co-agonist (Tong *et al*., 2008). After glutamate release, NDMARs are calcium permeable allowing an influx of calcium to occur leading to phosphatase and kinase activation favouring neurotransmission. In addition it allows the influx of sodium leading to depolarisation of the neuron. The most critical factor of activation of this receptor is the depolarisation of the neuron. Under resting conditions, a magnesium ion is bound preventing the activation of the receptor (Ascher *et al.*, 1988; Nikolaev *et al.*, 2012). Furthermore, this prevents any unwanted ions passing through into the neuron but the depolarisation results in magnesium removal. This removal allows calcium, sodium and potassium ions to pass depolarising the postsynaptic neurons (Wang *et al.*, 2011). These receptors have been studied extensively and have been shown to be involved in synaptic plasticity, memory and neurodegeneration (Cull-Candy *et al.*, 2001; Zhuo, 2009). Studies have also shown presynaptic NMDARs to exist where they function to control the release of neurotransmitter and also perform a more widespread function by regulating the signalling of neurons within the CNS (Duguid and Smart, 2009; Yan *et al.*, 2013).

1.3.4.2. AMPA receptors

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) have an affinity for glutamate which is lower than that of NMDAR but higher than kainate receptors. They are localised postsynaptically and consist of 4 subunits known as GluA1-4 which can assemble into either homomeric or heteromeric assemblies (Boulter *et al*., 1990). AMPARs are involved in fast excitatory synaptic transmission and the key feature that allows this fast transmission is the ability of the receptor to open and close rapidly. Unlike NMDARs, the majority of these receptors are calcium impermeable due to the presence of the GluA2 subunit however they are required for the initial excitatory signal when glutamate is released (Mayer, 2006). When glutamate binds, it activates the receptor permitting an influx of sodium and potassium which flows through the AMPARs resulting in the depolarisation of the neurons membrane allowing action potentials to carry on from neuron to neuron (Sprengel, 2006). Depolarisation of the neuron in turn results in the release of the magnesium ion bound to NMDARs allowing calcium influx through them. This allows AMPA receptors to play the roles involved in synaptic transmission which is mediated by glutamate and synaptic plasticity. Furthermore, the increase and removal of AMPAR at the synapses regulate synaptic plasticity through long-term potentiation and long-term depression, respectively (Bliss and Lomo, 1973; Lüscher *et al*., 1999; Emond *et al.*, 2010).

1.3.4.3. Kainate receptors

Kainate receptors have the weakest affinity for glutamate in comparison to NMDARs and AMPARs and are not as well understood as these other receptors due to their limited study. However evidence has shown them to have similar characteristics to AMPARs (Wilding and Huettner, 1997). The subunits consist of GluK1-5 and functional receptors form homomers but have the capability to make heterotetramers. Heteromers can be assembled if GluK4 or GluK5 if expressed with the other subunits however no function is present if expressed themselves. Like NMDARs, they are also localised presynaptically as well as postsynaptically (Chittajallu *et al*., 1999). Postsynaptically, they are involved in excitatory neurotransmission by eliciting a rapid, fast onset and desensitising response (Wondolowski and Frerking, 2009). However the presynaptic role is less well understood and has been suggested to function in the inhibitory response of neurotransmitter release through GABA (γ-Aminobutyric acid) release mediation as well regulating neurotransmitter release in an excitatory manner (Kane-Jackson and Smith, 2003; Kullmann *et al.,* 2005). One study showed that although kainate receptors are ionotropic, the action through which GABA release is mediated by presynaptic kainate receptors is metabotropic. The mechanism which this occurs was not shown to be through ion channel activity but actually through the involvement of a G protein causing a second messenger cascade resulting in the activation of phospholipase C (PLC) and protein kinase C (PKC), (Rodriguez-Moreno and Lerma, 1998).

1.3.5. Metabotropic glutamate receptors

Metabotropic glutamate receptors are found both presynaptically and postsynaptically in neurons which belong to a group of G-protein-coupled receptors that activate a cascade of events leading to protein modification and are involved in the perception of pain, learning and memory (Nicoletti *et al.*, 2011). The receptors make up three groups consisting of eight different types (mGluR1-8) that have roles in anxiety, learning and memory and their activation can either be excitatory or inhibitory. Group 1 (mGlu1 and mGlu5) are coupled to Gq/G_{11} whereas the remaining groups, group 2 (mGlu2 and mGlu3) and group 3 (mGlu4, mGlu7 and mGlu8) are classically coupled to Gi/Go (Simonyi *et al.*, 2010; Pitsikas, 2014). Research has shown that they modulate CNS function by controlling neuronal excitability and causing changes in synaptic excitability through release or inhibition of neurotransmitter and also mediate long term depression (Grueter and Winder, 2009).

1.3.6. Synaptic plasticity

Glutamatergic neurons can mediate many processes in the CNS vital for functioning such as the maturation of neurons, synapse formation and excitotoxicity (Van der Sluijs and Hoogenraad, 2011). However in relation to the hippocampus, glutamate neurotransmission is involved in the phenomena of synaptic plasticity which is the ability of synapses to strengthen or weaken depending on whether there is an increase of decrease in activity. Synaptic plasticity takes place in other regions of

the brain but is known to be one of the more important neurochemical foundations of learning, memory formation and retrieval and spatial recognition and is modulated by NMDARs and AMPARs (Bliss and Lomo, 1973; Jia *et al*., 1996; Kessels and Malinow, 2009). There are two mechanisms for synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD).

1.3.6.1. Long-term potentiation

In the 1800's, the discovery that neuronal number does not increase with age led to the suggestion that memories therefore couldn't form as a result of new neuron generation. This suggestion resulted in numerous scientists proposing that synaptic strengthening may be the mechanism for memory formation (Radwanska *et al.*, 2011).

Long-term potentiation is the strengthening of excitatory synapses caused by an increase in activity resulting in long lasting (minutes to months) transmission between neurons (Goh and Manahan-Vaughan, 2015). This mechanism was first discovered by Lomo when he conducted experiments on rabbits to determine if the hippocampus was linked to short-term memory. These experiments discovered the phenomenon, LTP, when a high frequency stimulus resulted in a long lasting increase in the response, termed excitatory postsynaptic currents (EPSCs), of postsynaptic cells to single-pulse stimuli (Bliss and Lomo, 1973). LTP was also observed in the amygdala, cerebellum and cerebral cortex. However in the context of memory, LTP is now known to be a key factor involved in the development of brain connectivity and

formation and storage of memories in the hippocampus (Bliss and Cooke, 2011). LTP can be NMDAR-dependent or NMDAR-independent but in the hippocampus where synaptic plasticity is robust, LTP, although not in the case of the mossy fiber pathway of the hippocampus, is generally NMDAR-dependent (Doherty *et al*., 2009). NMDAR-dependent LTP requires subsequent calcium influx along with sufficient depolarisation to remove the magnesium ion from the NMDA receptor and the neurotransmitter, glutamate, binding to the receptor. Early-form LTP (E-LTP) maintenance requires the activation of CamKII and PKC which translocate to the synapse and is required for AMPA receptor phosphorylation resulting in potentiation (Lisman *et al.*, 2012). This results is an increase in the receptors activity and the modulation of AMPAR surface expression at synapses (Isaac *et al*., 1995; Halt *et al.*, 2012; Zhang *et al.*, 2013). E-LTP is postsynaptic however increasing evidence suggests LTP could be expressed presynaptically through retrograde signalling (Bliss and Collingridge, 2013).

LTP stimulates an increase in AMPAR exocytosis which is required for increasing the cell surface population. The regulation of AMPAR expression occurs through AMPAR recycling and studies have shown the prevention of exocytosis leads to LTP inhibition (Malenka and Bear, 2004; Villers *et al*., 2012). Unlike E-LTP that does not depend upon protein synthesis, late LTP does. This form of LTP results from the activation of protein kinases, specifically mitogen-activated protein kinase (MAPK) and protein kinase m zeta (PKMʒ), during E-LTP and is required for its maintenance (Li *et al.*, 2014).

1.3.6.2. Long-term depression

In contrast to LTP, long-term depression (LTD) is the activity-dependent weakening of synapses in the hippocampus due to decreasing activity which can last hours. A low stimulation in addition to a smaller increase in intracellular calcium is required to induce LTD (Dacher and Nugent, 2011). Studies have suggested that LTD may be required for the removal of old memories and can be dependent on NMDARs and mGluRs. LTD mainly results from the decrease in postsynaptic receptor density through AMPAR internalisation and the receptor subunit GluA2 is required for this to occur (Lee and Kirkwood, 2011; Anggono and Huganir, 2012). A balance is required therefore without LTD, synapses would continue to strengthen resulting in encoded new information being inhibited (Turrigiano, 2008). Studies have shown that LTP and LTD can be prevented by the inhibition of NMDARs. Therefore LTP results from AMPAR recycling carried out by exocytosis of receptors to the synaptic surface whereas LTD results from AMPAR endocytosis (Biou *et al.*, 2008).

1.4. General introduction to the immune system

Organisms are constantly exposed to a variety of potentially dangerous pathogens that can present themselves and be transmitted through several routes including air-borne, direct/indirect and sexual contact and through blood or bodily fluids (Kilpatrick *et al.*, 2008; Unicomb, 2009; Fernstrom and Goldblatt, 2013). As pathogens can target multiple areas of the organism, for example the brain and skin, the immune system has

evolved to exist as a multifunctional defence mechanism to target and eliminate the threat through the production of cells and mediators relevant to the area of infection. The immune system is composed of two parts, innate and adaptive, where an intricate interplay exists between both allowing for harmful pathogens to be recognised and removed without the recognition and subsequent damage of tissue and commensal bacteria belonging to the host which itself are not threats (Bewick *et al*., 2009; Paulson *et al*., 2012).

1.4.1. The Innate immune system

The innate immune response has been conserved throughout evolution and is not only found in vertebrates but also in plants and invertebrates (Guo *et al*., 2009). It is the host's non-specific, first line of defence that acts within the first few hours of exposure to a pathogenic threat and uses a variety of factors to restrict and remove invading pathogens including bacteria, fungi, viruses and parasites without damaging resident microbiota (Allen and Sutherland, 2014; Guidry *et al*., 2014; Münz, 2014). These factors include a physical and chemical barrier such as the skin, production of mucus to trap pathogens or acid in the stomach to destroy the threat and a cellular component consisting of macrophages, neutrophils, endothelial cells and mast cells which can produce mediators, engulf cells in a process called phagocytosis and digest them or activate other cells. In addition the pathogen can be killed directly by soluble factors including complement proteins which create holes in a pathogens cell membrane (Gallo and Nizet, 2008; Muller *et al*., 2008; Hooper *et al*., 2012). Not only is the innate response the host's

first line of defence but it also functions to activate the adaptive immune response and prevent the threat becoming out of control by signalling to other immune cells to become involved (Bonilla and Oettgen, 2010; Schenten and Medzhitov, 2011).

1.4.2. Adaptive immune system

The adaptive immune response occurs only in vertebrates and is also known as acquired/specific immune response due to its capability of specifically recognising certain parts of a pathogen known as the antigen. Generally, the antigen expressed on a pathogen is a molecule such as lipopolysaccharide (LPS) or flagellin found on bacteria or single/double-stranded RNA belonging to viruses (Meyer et al., 2007; Kumagai and Akira, 2010). The response is mediated by T lymphocytes and B lymphocytes that function to amplify the innate response and the shaping of the immune response whether it be an antibody mediated response due to a greater preference for B lymphocytes or cell mediated due to T lymphocyte preference, is dependent on the innate response. In addition, this will also lead to immunological memory development, therefore if the same pathogen becomes a threat in the future, a known response to eliminate the threat will be produced (Narni-Mancinelli *et al.*, 2007; Wager *et al.*, 2011; Casadevall and Pirofski, 2012). However, these responses cannot recognise and act against pathogens without a specific mechanism enabling them to do so.

1.4.3. Pattern recognition receptors

Specific molecules native to pathogens are known as pathogen associated molecular patterns (PAMPs) and it is this specific part of the pathogen that allows the host to recognise them as being foreign and therefore a potential threat to the host. They are broadly shared by pathogens however the host response can distinguish between PAMPs and host molecules (Mogensen, 2009). These PAMPs can be proteins, carbohydrates, lipids and nucleic acids expressed throughout the pathogen's structure whether it comes from the surface of the pathogen or originates within it (Kawai and Akira, 2010). The mechanism allowing for an immune response to be mounted after determining a foreign body is enacted through pattern recognition receptors (PRRs) that are found on cells belonging to the host (Zhong *et al.*, 2009). PRRs include families of Toll-like receptors (TLRs), C-type lectin receptors, NOD-like receptors and RIG-I-like receptors and are classified into these families dependent on function, ligand specificity and localisation (Elinav *et al.*, 2011; Kawai and Akira, 2011; Loo and Gale, 2011; Osorio and Reis e Sousa, 2011). Amongst the several classes of PRRs, TLRs can mediate the recognition of these PAMPs leading to the initiation of a signalling pathway with the end result being the activation of the innate immune system.

1.5. Toll-like receptors

The Toll gene was first discovered in 1985 however its role was not in immunity but instead was identified to be part of the development of the dorsal-ventral region of the fruit fly, Drosophila melanogaster (Hashimoto *et al*., 1988). Although this original discovery was not related to immunity, subsequent studies led to the role of Toll signalling being shown to be involved in the immune response to pathogens (Lemaitre *et al.*, 1996; Fullaondo and Lee, 2012). Initially, genetic screening was carried out leading to the discovery of genes important in the dorsal-ventral region including Toll and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) homolog dorsal (Nüsslein-Volhard and Wieschaus., 1980; Belvin and Anderson, 1996).
In mammalian immunity, NF-κB was shown to play a role and therefore it gradually became evident that embryonic development of the fruit fly and the immune system may be analogous in ways. This was supported by a study carried in by Hultmark and colleagues in 1995, identifying Toll as an activator of the immune system. In addition, a human homolog of Toll was discovered and mapped shortly after (Rosetto *et al*., 1995; Taguchi *et al.*, 1996). Continuing on from these discoveries, shortly after in 1997, the first mammalian TLR was described (Medzhitov *et al*., 1997).

TLRs obtained their name due to their similarity to the protein which is coded by the Toll gene. They are a class of evolutionary conserved PRRs of which there are thirteen discovered to date. Ten TLRs are found in humans named TLR1-10 (Mai *et al.*, 2013). Furthermore, equivalent

forms of TLRs discovered in humans have not been identified in other mammals and it has been suggested certain mammals may express TLRs not found in humans (Li *et al.*, 2014). In general, their function is to be a key part of the innate immune system and have been identified as a central point in the protection of an organism against bacterial and viral pathogens (Areal *et al*., 2011). In addition they have been shown to allow immune cells to sense and act against potentially dangerous challenges from a wide variety of pathogens (PAMPs) but they can also respond to ligands involved in tissue damage (Shanker, 2010; Lewis *et al.*, 2013). In turn, TLR-mediated immune activation will then ultimately lead to an adaptive response resulting in pathogen elimination and protection of the host (Anderson, 2008). Furthermore, the adaptive immune response can also specifically recognise PAMPs through receptors present on T cells and B which are produced de novo (Iwaski and Medzhitov, 2010). PRRs are limited receptors whereas those involved in the adaptive immune system are unlimited which further emphasises how specific the adaptive response truly is (Silva, 2010; Zeng *et al*., 2012). This mechanism will lead to widespread activation of the immune system and ultimately result in the elimination of a pathogen causing infection. Furthermore, studies have shown that they are also involved in the development, physiology and metabolism of the host from the embryonic stage to the adult stage of the host (Okun *et al*., 2011).

1.5.1. Structure

TLRs are members of the type one integral transmembrane-glycoprotein receptor family, all of which have an extracellular N terminal ligand-domain for ligand recognition, a single transmembrane helix and a cytosolic Toll/IL-1R resistance (TIR) domain that is imperative for signalling (Godfroy *et al*., 2012). Depending of TLR subtype, the extracellular ligand binding domain/ectodomains (ECDs) which will either reside on the cell surface which responds to lipid and protein ligands or on the endosomal compartments which are activated by non-self nucleic acids (Botos *et al.,* 2011; Gay *et al.,* 2014). All ECDs of the TLRs have a leucine-rich domain and these leucine rich repeats (LRRs) are known to be the building blocks that construct the extracellular domain of the TLR and fold into a solenoid structure (Fig 1.5). This gives TLRs the required conformation to perform its function correctly by interacting with the ligand resulting in immune recognition (Palsson-McDermott and O'Neill, 2007; Fang *et al*., 2012). After a stimulus binds to the ECD, it will induce dimerization of the TIR domains and these TIR domains will act as a signalling platform for adaptor recruitment and subsequent signal transducers. The adaptor proteins contain TIR domains which specifically engage with the receptor dimers through TIR-TIR interactions. Activation of TLRs results in the ectodomains coming into close proximity by juxtamembrane sequences which are connected to the transmembrane helix. The structure of the TIR domains commonly consists of an α/β fold with parallel β-strands at the core and α-helices surrounding it. These are all connected by loops and the most recognised being the BB loop that has an extremely important role in signal

transduction (Gay *et al.*, 2014). Interestingly, TLR3 is the only receptor that contains an alanine in the BB loop rather than a proline which exists for the other TLR. This has been shown to be important for adaptor specificity (Verstak *et al.*, 2013).

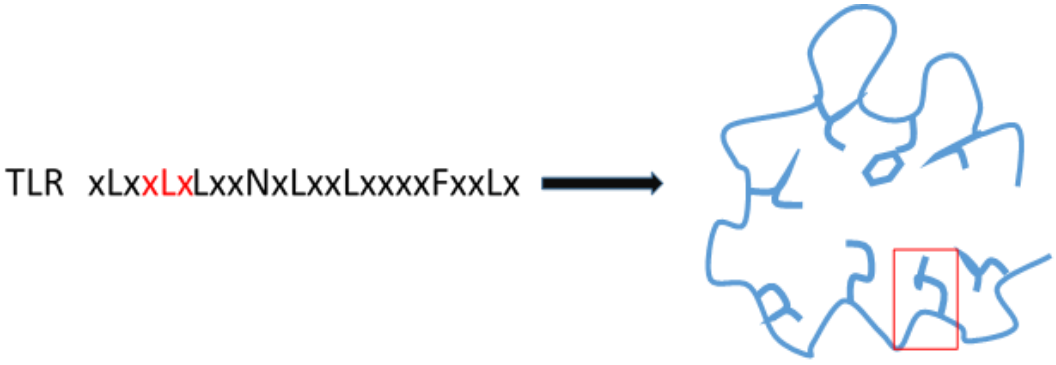


Figure 1.5: TLR3 consensus sequence of LRR and the sequence shown as an LLR loop of human TLR3.

If the TLR is one of either TLR3, 7, 8 or 9, which reside in endosomes within a cell and recognise nucleic acid PAMPs, the transmembrane protein known as UNC93B, which is mainly composed of hydrophobic residues, will direct the receptor to the endosome whereas the remaining TLRs will be trafficked to the cell surface (Kim *et al*., 2009). The TLRs can be grouped into 6 subfamilies based on the homology of their sequences, where the subfamilies interact to form signalling complexes to allow ligand recognition (Table 1.1). The TIR domains dimerize in response to dimerization of ectodomains induced by a ligand which in turn signals the immediate threat a pathogen poses and subsequently activates the relevant signalling pathway (Matsushima *et al.*, 2007; Gay *et al.*, 2014).

Interestingly, one study showed that a patient completely deficient of TLR3, who in childhood, developed herpes simplex virus 1 encephalitis (HSE) remained resistant to other infections. In addition, human TLR3 was shown to not be required in host defence against other infections suggesting that in patients with TLR3 deficiencies, other antiviral immune pathways or dsRNA-responsive pathways independent of TLR3 may compensate for the loss of TLR3 function and therefore contribute to controlling viruses other than HSV-1 (Guo *et al.*, 2011).

TLR	Localisation	PAMP	Source of PAMP
1/2	Plasma membrane	Triacyllipopeptides	Bacteria
2/6	Plasma membrane	Diacyllipopeptides	Bacteria
3	Endosome	Double-stranded RNA and Poly I:C	Virus
4	Plasma membrane	Lipopolysaccharide (LPS)	Bacteria
5	Plasma membrane	Flagellin	Bacteria
7/8	Endosome	Single-stranded RNA	Virus
9	Endosome	UnmethylatedCpGDNA	Bacteria and viruses
1/10, 6/10 and 1/6/10	Unknown	Unknown	Unknown

Table 1.1: The localisation of each TLR, whether they function alone or in conjunction with other TLRs, what PAMP they recognise and what pathogen the PAMP belongs to (adapted from Kumagai and Akira, 2010).

1.5.2 Signalling pathways

As indicated earlier, TLRs are grouped into subfamilies and they are characterised by the adaptor molecules they interact with. Receptor-ligand interaction enables signalling pathway activation resulting in the production of the appropriate mediators to activate the immune system (Mogensen, 2009). There are two signalling pathways allowing for the propagation of signals that derive from their respective TLRs through the use of their adaptor molecules. The MyD88-dependent pathway uses the intracellular adaptor molecule, MyD88 and the MyD88-independent pathway uses TIR-domain-containing adapter-inducing interferon-β (TRIF) as the adaptor molecule (Bagchi *et al.*, 2007; Petnicki-Ocwieja *et al.*, 2013). Studies have shown that the majority of TLRs act through the MyD88-dependent pathway however TLR3 is the only receptor that can act through the MyD88 independent pathway alone. TLR4 is the only receptor that can act via both pathways (Tesse *et al.*, 2011).

1.5.2.1 MyD88 dependent pathway

The MyD88 dependent pathway occurs through the interaction of the TIR domain of the TLR associating with MyD88. MyD88 also has a TIR domain at its C terminus enabling it to become activated when the PAMP binds to the receptor (Fekonja *et al.*, 2012). A receptor complex is then formed when Interleukin-1 receptor-associated kinase 4 (IRAK4) is recruited by Myeloid differentiation primary response gene 88 (MyD88) by death domains present in both molecules and this complex then helps to phosphorylate IRAK1. In addition, the receptor complex is

also required for the degradation of the IRAK1 protein (Suzuki *et al*., 2002; Avbelj *et al*., 2011). TNF receptor-associated factor 6 (TRAF6) is then recruited and activated resulting in the dissociation of IRAK1 and TRAF6 from the receptor complex and these then interact with TGF-beta activated kinase 1 (TAK1) and the TAK1 binding proteins TAB1 and TAB2 to form a membrane bound complex. TRAF6 then undergoes numerous ubiquitination reactions resulting in the membrane bound complex being activated. TAK1 and TAB2 are phosphorylated resulting in the dissociation of the complex from the membrane where TAK1 phosphorylates targets called IKKs downstream (Jiang *et al*., 2002; Flannery and Bowie, 2010; Zeng *et al*., 2012). NF-κB is a protein complex that controls the transcription of immunoregulatory genes. Inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ) is a protein that is bound to the transcription factor NF-κβ forming the complex into an inactive state through preventing its translocation and activation into the nucleus. It is rapidly activated because it does not require protein synthesis for activation therefore can respond first to stimuli. Iκβ is phosphorylated and degraded by an IKK complex when the stimulus arrives which activates NF-κB allowing it to induce the expression pro-inflammatory cytokines and chemokines such as TNF-α and IL-8 (Regnier *et al.,* 1997; Sun and Andersson, 2002).

1.5.2.2 MyD88 independent pathway

As already stated, TLR3 is the only receptor that acts through the MyD88-independent pathway through the TRIF adaptor molecule however TLR4 can also act through the pathway using TRIF-related

adaptor molecule (TRAM) where IFN leads TLR4 to signal via the TRIF pathway (Gangloff, 2012; Verstak *et al*., 2013). Through the activation of IRF3, TRIF was established as the adaptor protein that controls TLR3 and TLR4-mediated IFN production (O'Neill and Bowie, 2007). TRIF consists of an α-helical N terminal domain followed by binding sites for effector proteins downstream including TRAF2 and TBK1. Furthermore, the C terminus contains a TIR domain and a receptor interacting protein (RIP) homotypic interaction motif (RHIM) domain. It has been shown that the N terminus binds to the TIR domain preventing the binding sites being accessed in cells that are unstimulated. However in stimulated cells, TRIF binds to active TLR3 by its TIR domain which removes that N terminus that was originally bound and enables downstream effector proteins to bind. TRIF has been shown to mediate 3 distinct signalling pathways for TLR3. The first involves IRF3 and/or IRF7 to become activated via TBK1 and TRAF3 binding to TRIF. IRFs then bind to interferon sensitive response element (ISRE) on target genes to produce type one interferons (O'Neill and Bowie, 2007; Youn *et al.*, 2009; Gay *et al.*, 2014). Secondly, RIP1 can bind to the RHIM domain of TRIF resulting in FADD-dependent apoptosis and thirdly, NF- κB activation resulting in cytokine and chemokine production (Fig 1.6).

Ultimately, the TLR3 signalling pathway results in the production of pro-inflammatory cytokines and type one interferons which aim to protect the host against invading viral pathogens that pose a threat to the host's health and amplify the anti-viral immune response. Furthermore, chemokines recruit cells to the site of infection which will either lead to further cell recruitment or further pro-inflammatory cytokine release which further amplifies the anti-viral immune response (Jiang *et al.*, 2004; Famakin *et al.*, 2011).

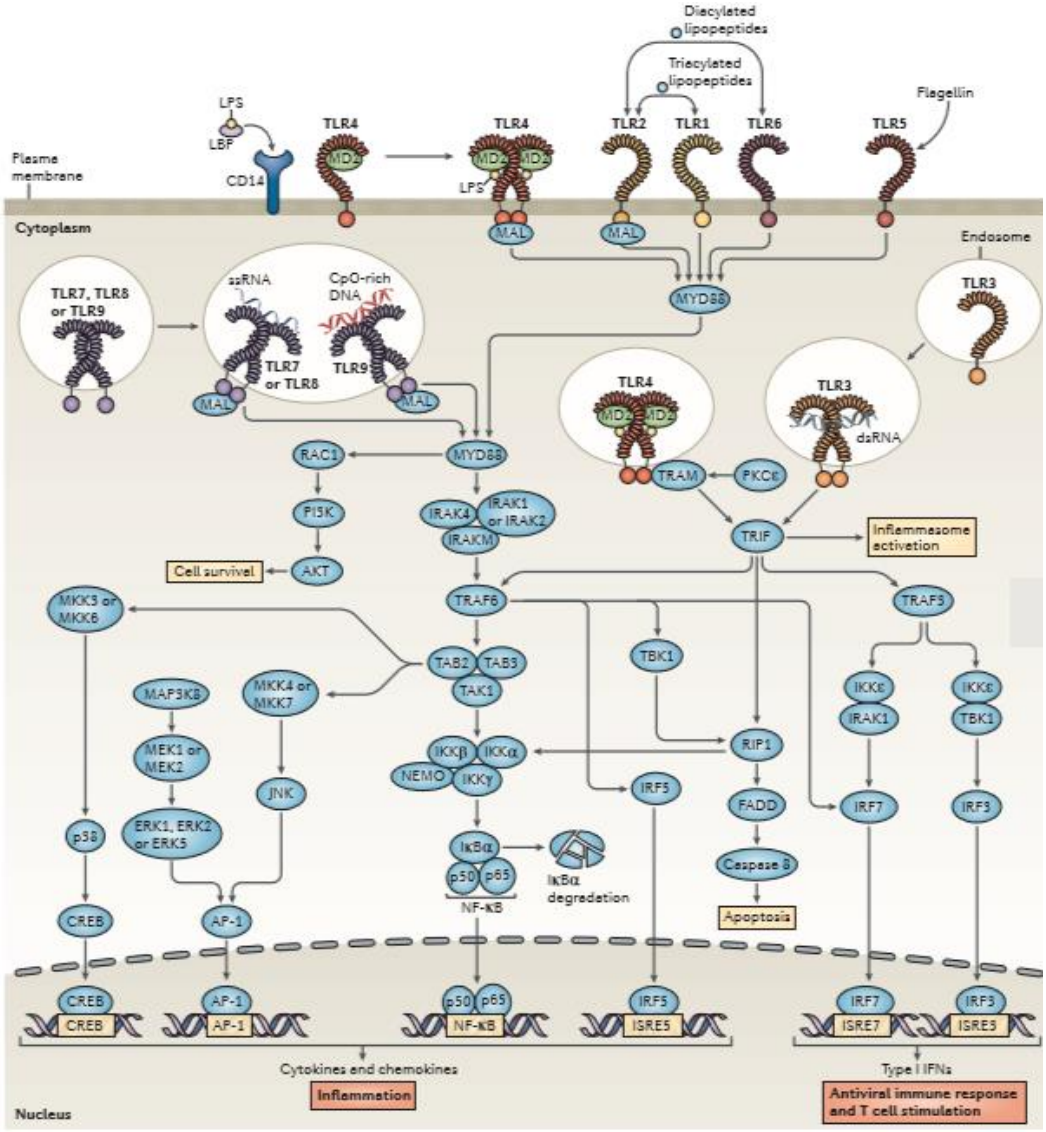


Figure 1.6: TLR signalling pathways (Gay *et al.*, 2014).

Cell type expression

When a pathogen threatens the health of a host, the host relies on the respective TLR to be activated which in turn activates the immune

system allowing the required immune function to remove the threat. There is differential expression of TLRs throughout the body along with cellular locations of TLRs which are mainly composed of antigen presenting cells (APCs). APCs including macrophages, dendritic cells and B cells function to express an antigen from a pathogen on their own surface to alert other cells allowing the immune response to be activated (Mogensen, 2009; Kawai and Akira, 2011). Depending on the pathogen, whether it is bacteria, a virus, protozoa or fungi, respective TLRs will be activated within specific cells allowing a variety of cell types (immune and non-immune) to respond and recognise specific PAMPs from the pathogens (Meyer *et al.*, 2007).

TLRs can be expressed in immune cells including macrophages, natural killer cells, dendritic cells, neutrophils, T cells and B cells and can also be present in non-immune cells including epithelial cells, endothelial cells and cells of the brain to name a few (Kaisho and Akira, 2006; Ospelt and Gay, 2010; Yang *et al.*, 2011). Not only is the expression of TLRs specific to different types of cells, their activation also relies on where the invading pathogen localises to. In mammals, the majority of TLRs are expressed on the plasma membrane of cells where they recognise pathogens that localise in the extracellular region whereas PAMPs such as a virus DNA or RNA that localise and infect cells within the cell itself are recognised by TLRs present on the endosomes (Simone *et al.,* 2011). TLRs expressed on the plasma membrane of cells function to recognise extracellular pathogens that do not enter the cell in order to use the hosts machinery to its own advantage (Chaturvedi and Pierce, 2009). These pathogens include bacteria and fungi where a component of their cell wall is recognised by TLRs including TLR 1,2,4,5 and 6, which

subsequently allows the activation of signalling pathways leading to the initiation of the immune response (Akira *et al*., 2006; Simone *et al*., 2011). In addition, certain pathogens can be recognised by more than one TLR and TLRs themselves can recognise structurally unrelated ligands from multiple pathogen groups (Flannery and Bowie, 2010). In contrast, TLRs 3, 7, 8 and 9 are found on intracellular endosomes and function to recognise nucleic acids from viruses leading to their elimination through appropriate cell activation and mediator release (Jack *et al*., 2005; Kumagai and Akira, 2010).

Furthermore, it has unexpectedly been found that TLR interactions with their ligands are more complex than originally thought as not only do they recognise PAMPs but following tissue injury, TLRs may recognise and interact with host proteins (Piccinini and Midwood, 2010). It has also been shown that TLRs can interact with other innate immune receptors including scavenger receptors which results in the phagocytosis of bacteria by the scavenger receptors and G-protein coupled receptors (GPCR) resulting in the decrease of GPCR desensitisation which augments polymorphonuclear phagocytes through LPS-TLR4 mediated signalling (Mukhopadhyay *et al.,* 2002).

1.5.3 Endogenous activators

In addition to microbial PAMPs being ligands of TLRs, endogenous ligands have also been known to interact with TLRs and are potent activators of innate immunity. During infection, TLRs are activated by microbial PAMPs with the aim of removing the pathogenic threat from

the host so that they remain healthy. However endogenous activators have been shown to interact with TLRs mainly in non-infectious environments (Yu *et al.*, 2010) such as tissue injury and autoimmune diseases inducing a sterile inflammatory response (Tsan and Gao., 2004). Endogenous ligands are often referred to as alarmins as they are early warning signals to the innate and adaptive immune response (Oppenheim and Yang, 2005; Saïd-Sadier and Ojcius, 2012). It has been suggested that for the immune system to be activated, there has to be the presence of both PAMPs and danger signals because not only does the danger signals allow the recruitment of more cells through the immune system sensing an infection, it also allows the repair process to begin (Medzhitov, 2008).

The endogenous activators are subgroups of a larger category termed DAMPs. They are tissue or cells derived from the host and the majority are components of the extracellular matrix including oligosaccharides, fibronectin and fibrinogen or mediators of the inflammatory response including heparin sulphate and heat-shock proteins (Millar and Murrell, 2012; Kelsh and McKeown-Longo, 2013). These endogenous ligands activate TLRs in many pathological states and during these states, they are released from dying cells or tissues that have been damaged or from activated cells (Pollalen *et al.,* 2009). There are different endogenous ligands for TLRs for example, hyaluronan activates TLR2/4 and RNA from necrotic cells activates TLR3 (Yu *et al.,* 2010). A study has suggested that TLR3 regulates the amplification of the immune response by potentiating or aggravating inflammation that pre-exists and when independent of viral activation, serves as an endogenous sensor for necrosis (Cavassani *et al.,* 2008). Following on from the study, evidence

is accumulating to show that in some pathological conditions, the inflammatory signals can be activated in the absence of infection. TLR signalling triggers transcription of pro-interleukin-1β and pro-IL-18 that are then processed by the inflammasome into their activated state. The inflammasome is a multiprotein complex that provides a molecular platform allowing for the activation of the inflammatory caspases (Sutterwala *et al.,* 2009). The interleukins are involved in the pathophysiology of various neurodegenerative diseases including Alzheimer's disease and CNS infections including bacterial meningitis and have been shown to influence the development of these diseases and regulate adaptive immunity induction (Hanamsagar *et al.*, 2012).

As already stated, Toll in Drosophila play a role in nervous system formation and immunity however recently, specific subtypes of Toll have been shown to be involved in neurotrophism. Neurotrophins are essential growth factors in crucial development processes such as proliferation, synaptic plasticity, apoptosis, differentiation and survival (Bartkowska *et al.,* 2010). A study showed that throughout development, Toll-6 and Toll-7 are expressed in the CNS where they function in regulating locomotion and neuronal survival. Furthermore, the study showed that neurotrophins called DNT1 and DNT2 directly interact with Toll-6 and Toll-7 therefore these toll receptors mediate neurotrophic responses to endogenous neurotrophic ligands (McIlroy *et al.,* 2013). Although Toll and TLRs are distinct from each other, they have both been shown to be involved in nervous system development and immunity. In the mammalian CNS, many of the endogenous ligands are unknown however a few have been shown to interact with specific

TLRs. Whether TLRs in the mammalian CNS interact with neurotrophic factors is as yet unknown and requires further investigation.

1.6. The CNS and the immune system

In the past, the brain was considered to be an immune privileged site and its activities mutually exclusive to that of the immune system. However it is now established that the involvement of immune cellular and molecular substrates support the defence of the CNS and that actually, immune cells are present in the CNS where immune responses do occur when required (Engelhardt and Coisne, 2011; Ransohoff and Brown, 2012). Furthermore, the CNS undergoes constant surveillance and it was accepted that if an immune response was mounted, immune cells would infiltrate the CNS stimulating an inflammatory reaction. Entry would occur via the breakdown of the BBB as a well-known characteristic of the CNS was its lack of a lymphatic drainage system (Carson *et al.*, 2006; Libbey and Fujinami, 2014). However recently, Louveau and colleagues discovered in their search for T cell gateways, functional lymphatic vessels in the CNS expressing hallmarks similar to endothelial cells of the lymphatic system. They were discovered lining the dural sinuses connected to lymph nodes allowing immune cells to be carried from the cerebrospinal fluid. This exciting evidence may give new insight into the CNS-immune system interactions and therefore, CNS defence against pathogens (Louveau *et al.*, 2015). Defence of the CNS requires a rapid immune response to be mounted through cell activation to prevent further exacerbation of the threat and robust antibody production by B cells to neutralise infection (Griffiths *et al.*, 2010).

Minimal damage is favourable and key to an effective immune response, however the activation of the immune response may also damage healthy host cells and tissue causing impairment in CNS function. Furthermore, host tissue damage and disease states causing impairments may also induce transient or enduring behavioural changes in the host and lead to the progression of neurodegenerative diseases (Amor *et al.,* 2010; Chaplin, 2010).

1.6.1. Interplay between the CNS and the immune system

In the CNS, there is an intricate interplay between the CNS and the immune system. TLRs have been best known for their damaging effects when activated and they not only recognise PAMPs but are also capable of recognising DAMPs (Olivieri *et al.,* 2013). TLR activation in response to these ligands released via injury and stress, leads to the activation of the immune response. Evidence has been accumulating to suggest that TLRs play a major role in the context of brain infection and injury (Mallard, 2012). They are also proposed to be implicated in neurodegenerative disorders such as Alzheimer's disease, autoimmune neuropathy from multiple sclerosis (MS) and have been shown to cause virus infection-induced neuroinflammation. For example, TLR3 activation in a mouse model of herpes simplex virus 1 (HSV-1) that were pre-treated with the TLR3 agonist, poly I:C, before infection with the virus led to a lower virus load and a higher survival rate. This was due to the early expression of immune genes in the brain that were produced to aid in the elimination of the virus (Arroyo *et al.,* 2011; Reinert *et al.,*

2012). There has also been some supportive evidence of neuroprotection (Hanisch *et al.*, 2008).

TLR1-9 have been shown to be expressed in cells throughout the brain (Table 1.2), and when activated this results in the release of inflammatory mediators and an increase in the permeability of the blood brain barrier allowing an influx of immune cells into the CNS to aid in eliminating infection (Carty and Bowie, 2011). Furthermore, TLRs, especially TLR3 are now known to be involved in not only immune responses but also in non-immune responses such as neurogenesis and the development of the brain (Lathia *et al.*, 2008; Okun *et al.*, 2010; Hanke and Kielian, 2011). There is limited knowledge regarding TLR3 function in the CNS however what is known allows for some intriguing speculations to be made and for this reason, the rest of this section will focus on TLR3 and the current knowledge on TLR3 under homeostatic and pathophysiological conditions in the CNS.

Cell type of CNS	**TLR expressed**
Microglia	1-9
Astrocyte	3 and 4
Oligodendrocyte	2 and 3
Neuron	3

Table 1.2: Cell types in the human CNS expressing TLRs (adapted from Carty and Bowie, 2011).

1.6.2. The role of TLR3 in the CNS and in injury

Under resting conditions, TLRs are expressed in relevant cell types of the brain and function mainly to alert the immune response to any threat that may cause infection by recognising PAMPs from pathogens (Kigerl *et al.,* 2014). In agreement, studies have indicated that TLR3 is highly expressed in the CNS under resting conditions and therefore may have a critical role in immunity of the brain and also brain homeostasis (Carpentier *et al*., 2008). TLR3 is considered to be an important receptor within the CNS because of its anti-viral properties. The receptor is classically localised intracellularly in membrane bound compartments called endosomes as viral PAMPs have been shown to originate within the cell. However within cells of the CNS, increasing evidence shows expression of TLR3 on the plasma membranes of neurons and astrocytes (Wang *et al.,* 2009).

Microglial cells have been shown to express TLR3 on intracellular endosomes and as they are the CNS immune cells, they combat many infections (Suh *et al.,* 2009). Limited evidence exists regarding TLR expression on oligodendrocytes or what there function is when activated, however it has been shown that human oligodendrocytes express TLR2 and TLR3 with supporting evidence suggesting that the agonists of these receptors regulate their survival and differentiation and therefore the formation of myelin (Bsibsi *et al.,* 2006). Human astrocytes have been reported to constitutively express TLR3 on the cell surface and also intracellularly (Li *et al.,* 2012). The receptors respond to neuroinflammation and when activated, respond in a neuroprotective

manner by producing neuroprotective, growth and differentiation mediators (Yuan *et al.,* 2010). A study showed that the mediators released by astrocytes leads to the inhibition of astrocyte growth, enhancement of neuronal survival and the promotion of endothelial cell growth in organotypic human brain slice cultures (Table 1.3), (Okun *et al.,* 2009). In permissive environments, neurons are capable of growing new axons however there is limited ability to regenerate axons when CNS injury occurs (Yiu and He, 2006; Ferguson and Son, 2011).

In drosophila, axonal guidance of motor neurons occurs when muscles express the activated Toll gene. This then led to the possibility that in the mammalian CNS system, axonal growth may be regulated by TLR expression (Rose and Chiba, 1999). One study showed that axonal growth from dorsal root ganglion (DRG) explants was negatively regulated in the presence of poly I:C overnight. Acute poly I:C also caused a four-fold increase in the growth cone collapse and irreversibly inhibited neurite extension. Mice were also injected with poly I:C which resulted in deficits in sensory-motor development and when the neurons were examined, it showed an inhibition on the growth of axons (Cameron *et al.,* 2007) therefore prevention of axonal growth seems to be limiting the regeneration and repair of the CNS.

Neuroprotective mediator	Function
Brain derived neurotrophic factor	Protects neurons from apoptosis and infectious damage and regulates dendrite formation
Neurotrophin 4	Neuronal survival, differentiation and maturation
Pleiotrophin	Survival of glial cells and neurons
Ephrin type B receptor 1	Migration guidance and promotes new neuron connectivity
TGF-beta2	Reduces demyelination from virally infected mice, protects neurons and inhibits astrocyte proliferation
Ciliary neurotrophic factor	Promote remyelination
Leukemia inhibitory factor	Promote remyelination

Table 1.3: Mediator release from astrocytes due to TLR3 activation and their functions (adapted from Bsibsi *et al.*, 2006).

1.6.3. The role of TLR3 in the CNS during infection

Following on from TLR3 function under homeostatic conditions, it has been widely shown that it plays a major role in immune activation under pathophysiological conditions (Ménager *et al.*, 2009). TLR3 is thought

to be a major component in mediating viral infections because it recognises double stranded RNA which is a common by-product generated when viruses replicate (Lai et al., 2011; Thompson *et al.*, 2011). Microglial cells are the CNS immune cells which scavenge for threats and debris and amongst recognising many viral infections, TLR3 has the unexpected ability to recognise Escherichia coli, a bacteria, and removes it through release of cytokines and chemokines which in addition lead to enhanced microglial phagocytosis of the pathogen (Ribes *et al.,* 2010). Furthermore, TLR3 activation was discovered to induce the apoptosis of oligodendrocytes and may indicate a role for the receptor in the immune response which may contribute to pathological conditions (Bsibsi et al., 2012). Pathological conditions also result in the activation of TLR3 on astrocytes to induce a protective response to infection. TLR3 on astrocytes respond to neuroinflammation caused through recognising infection and results in a neuroprotective response. This response involves the release of soluble molecules with neuroprotective properties (Steelman and Li, 2011). Although TLR3 activation has been found to be protective against many viruses and infections such as West Nile Virus through IRF3 production and Herpes simplex virus 1 to name a few (Daffis *et al.,* 2008), it can also lead to the progression of infection and/or disease as shown by Jin and colleagues. Their work showed that there was efficient protection against a neurovirulent strain of Theiler's murine encephalomyelitis virus (TMEV) infection. In contrast, this wasn't the case for a less virulent strain of TMEV and led to a higher viral load and disease progression. In addition, they suggested that TLR3 activation during infection is protective whereas activation of the receptor prematurely results in an increase in viral load leading to a pathogenic outcome therefore the

response by cells must be highly regulated to prevent neuronal damage and loss (Jin et al., 2011).

Furthermore, infections causing TLR3 activation have been shown to induce sickness-like behaviour as a result of proinflammatory changes in the CNS. These changes result from infections that have occurred in the periphery causing cytokine and bacterial toxins to be released. These then deliver the information that an infection is present to the brain communicating through humoral and neuronal routes (McCusker and Kelly, 2013). The sickness-like behaviour is composed of adaptive behavioural changes including fever, lethargy and loss of concentration resulting in depressive-like activity and anxiety (Cunningham *et al.*, 2007). It has been suggested that these behavioural changes are important to understanding depression (Maes *et al.*, 2012; Murray *et al.*, 2015).

Research has shown that during infection, cytokines, antibodies and activated T cells can disrupt brain function (Diamond et al., 2009; Miller, 2010). In agreement, TLR3 activation can modulate neuronal network excitability which may explain the behavioural changes induced during sickness like behaviour (Costello *et al.*, 2013). Furthermore, it is intriguing to speculate that this modulation could possibly be a protective mechanism during infection. Behavioural changes seen during infection can not only affect the host directly, but can also affect an unborn foetus during maternal infection. Maternal viral infection during pregnancy is associated with increased incidence of psychiatric disorders such as schizophrenia (Arrode-Brusés and Brusés, 2012). In addition to fighting infections, TLRs and immune activity have been implicated in

diseases of the CNS. They can exacerbate the condition via overstimulation of the immune system resulting in the overactivation of mediator release (Horton *et al.*, 2010).

1.6.4. The role of TLR3 in neurological disorders and neurodegenerative diseases

The active immune system and inflammation has now been accepted to be linked with neurodegenerative diseases and certain neurological disorders. However, whether inflammation is a direct cause or whether it is activated in response to the disease is still poorly understood (Bettcher and Kramer, 2013). Certain TLRs have well-established functions in specific diseases however our knowledge of TLR3 and its role is still limited. Epilepsy is one of the most prevalent neurological disorders with an association with inflammation. One study recently showed that WT TLR3 and knockout TLR3 mice developed spontaneous seizures in response to the anti-cholinergic compound pilocarpine however these epileptic seizures were prolonged and more frequent in WT mice. This suggests a possible role of TLR3 in the induction of epilepsy (Benninger *et al.*, 2014). In agreement, another study showed that mimicking a systemic viral infection by using poly I:C in female prion-diseased C57BL/6 mice (ME7 model of prion disease) resulted in an increased inflammatory response further exacerbating the disease (Field *et al.*, 2010).

In addition to exacerbating a response, TLR3 has been shown to also have the ability to induce protection. A protective role of TLR3 in

astrocytes has been proposed in patients that have MS, an autoimmune disease that leads to the destruction of oligodendrocytes and demyelination of neurons. The expression of TLR3 in MS lesions tends to be more concentrated at the late stage of the disease suggesting a role in promoting repair than exacerbating the inflammation already present through release of anti-inflammatory cytokines by restimulated T_H2 cells and neurotrophin production (Nair *et al*., 2008). Lifestyle choices can also play a role in TLR3-induced neuroinflammation and degeneration which may also increase the risk of neurodegenerative diseases. Research proves this by showing excessive alcohol consumption increased blood and brain TNF-α and brain cytokines that coincided with activated microglia. This response occurred via TLR3 activation resulting in inflammation (Bhat, 2010). Furthermore, markers of neurodegeneration were found to be present and when the systemic response subsided, a persistent neuroinflammatory response existed (Qin and Crews, 2012). As previously mentioned, there is limited knowledge of the role TLR3 plays in neurodegenerative diseases and neurological disorders however all studies are consistent in showing that TLR3 is involved in the neuroinflammatory response. Mounting evidence suggests that TLR3 and immune system molecules play other roles in the function of the brain, including specific areas, for example, the hippocampus (Okun *et al.,* 2011).

1.7. The hippocampus and TLR3

The hippocampus is an extremely important region of the brain located under the cerebral cortex in the temporal lobe. One exist on each side of the brain in humans and many other vertebrates and is involved in spatial memory and navigation, consolidating information from short to long-term memory and emotions (Scoville and Milner, 1957; Kesner and Hopkins, 2006). It is composed of the dentate gyrus, the CA1-4 zones densely composed of pyramidal neurons, the subiculum and the hippocampus proper (Clark and Squire, 2013). It is frequently applied as a neurophysiological model system and a great tool for studying many neurodegenerative diseases, for example Alzeihmers's disease, as the hippocampus is one of the first areas to be damaged in this disease (Mu and Gage, 2011).

Mounting evidence suggests that TLR3 and immune system molecules can be mediators of learning, memory, and synaptic plasticity. TLR3 has been shown to be widely expressed in cells of the hippocampus and is expressed in the CA1 subregion and dentate gyrus (Okun *et al.*, 2010; Okun *et al*., 2012). TLR3 activation has been shown to inhibit memory retention, adult neurogenesis, neural plasticity, hippocampal dependent learning and AMPA receptor expression in the hippocampus (Okun *et al*., 2011; Cameron *et al.*, 2007). Previous data showed TLR3 appears to dampen hippocampal-dependent learning and memory in normal mouse brains whereas TLR3 KO's showed improvement in hippocampal memory. This single study showed that KO mice spent more time searching for the platform in the Morris Water Maze compared to WT

mice and the KO mice performed better when locating the platform that had been changed to another quadrant. In addition, mice given poly I:C by CNS infusion did not perform as well as the control mice in finding the changed location of the platform. The TLR3 knockout mice also spent more time observing the novel object than those with TLR3 showing that TLR3 negatively regulates learning and memory of the mice. Furthermore, this was shown by TLR3 reducing neurogenesis and AMPAR expression in KO mice having a greater surface expression of the receptors at the surface in comparison to the WT. It has been speculated that other activators such as endogenous activators may be involved (Okun *et al*., 2010). Furthermore, a study looking at TLR3 in pruritis showed regulation of neuronal excitability and synaptic transmission. In conclusion, mounting evidence shows that TLR3 activation affects neuronal excitability and transmission through alterations in spontaneous action potential firing, AMPAR surface expression and sodium channel function.

1.8. Working hypothesis: aim and objectives

Extensive studies have been carried out on TLR3 and its function in immunity and in the CNS (Carpentier *et al*., 2008). However knowledge of TLR3 and its function and mechanism through which it acts within the CNS on synaptic plasticity, memory and learning is still very limited (Cameron *et al*., 2007; Okun *et al*., 2012). Therefore, this project will investigate the role of TLR3 activation in the modulation of hippocampal function that may provide key evidence underlying behavioural changes in the CNS mediated by viral infections.

Hence, the hypothesis of this research is that TLR3 modulates hippocampal function by reducing synaptic activity. This hypothesis will be tested by addressing the following specific questions.

1. What is the cellular localisation of TLR3?

2. Does TLR3 modulate synaptic activity?

3. What mechanisms underlie the effects of TLR3 activation on synaptic activity?

2. MATERIAL AND METHODS

2.1. Materials

COMPANY	MATERIALS
Sigma-Aldrich (Poole, UK)	D-glucose, sodium chloride (NaCl), ethylene-di-amine tetra-acetic acid (EDTA), ethylene glycol tetra-acetic acid (EGTA), paraformaldehyde (PFA), bovine serum albumin (BSA), phosphate buffer tablets (PBS), methanol, ethanol, papain, Triton X-100 solution, hydrochloric acid (HCl), adenosine triphosphate (ATP), guanosine-5'-triphosphate (GTP), potassium methyl sulphate ($KMeSO_2$), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), anti-β-III tubulin, cytosine β-D-arabinofuranoside (AraC), poly-L-lysine hydrobromide, anti-microtubule-associated protein (MAP)-2, polyinosinic:polycytidylic acid (Poly I:C) and lipopolysaccharide (from Salmonella Minnesota)
	Alexa Fluor anti-rabbit 488/555 IgG, Alexa Fluor anti-mouse 488/555 IgG,

Invitrogen (Paisley, UK)	Alexa Fluor anti-chicken 488/555 IgG, Alexa Fluor anti-sheep 555 IgG, B-27, L-glutamine, Neurobasal-A medium, foetal bovine serum (FBS), minimum essential medium (MEM) and lipofectamine 2000
VWR International Ltd (UK)	Calcium chloride ($CaCl_2$), magnesium sulphate ($MgSO_4$), potassium chloride (KCl), sodium dihydrogen phosphate (NaH_2PO_4), sodium hydrogen carbonate ($NaHCO_3$), sucrose, coverslips (round, 13mm, thickness No.1, Boroscillate Glass), microscope slide (superfrost, 76x26x1mm, white) and pasteur pipettes (plain glass, 150mm)
Abcam (Cambridge, UK)	Tetrodotoxin (TTX) and anti-TLR3
Ascent Scientific (Bristol, UK)	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) and DL-2-Amino-5-phosphonovaleric acid (DL-AP5)
Merck Millipore (Germany)	Anti-O4, N-terminal anti-GluA1, anti-Glial Fibrillary Acidic protein (GFAP) and Millex syringe-driven filter unit (0.22µm)
Wako Chemicals GmbH (Germany)	Anti-Iba1 rabbit
Invivogen (UK)	Polyinosinic:polycytidylic acid (HMW, poly I:C)

Clontech (Mountain View, USA)	Anti-green fluorescent protein (GFP)
Vector Laboratories (Peterborough, UK)	Vectashield with DAPI
Harvard Apparatus Ltd (UK)	Borosillicate glass micropipette (1.5mm OD x 0.86mm LD)
BD Biosciences (UK)	Cell culture dishes (35mm, TC treated)

2.2. Methods

2.2.1. Animals

One to two day old Sprague Dawley rat pups were obtained from in-house colonies maintained in the Biological Procedures Unit at Strathclyde University. All animal care and experimental procedures were in accordance with the guidelines of the UK Home Office (UK Home Office Schedule 1 guideline) under the agreement and authority of the UK Animals Act, 1986.

2.2.2. Primary hippocampal culture

2.2.2.1. Preparation of primary hippocampal culture

Coverslips previously autoclaved for sterility were coated in filter sterilized poly-L-lysine (0.1%) for 1 hour and then were washed in autoclaved sterile water for 10 seconds. They were left to dry on sterile

tissue paper prior to being plated and 3 coverslips were placed per petri dish (33mm). Hippocampal culture media (97% fresh Neurobasal A media, 2 % B-27 supplement and 1% 200mM stock concentration L-glutamine) was prepared and filter sterilised. In addition 1.5% papain and 1% bovine serum albumin (BSA) was prepared in 5ml and 6ml sterile buffered enzyme solution, respectively (in g/500mls: NaCl 3.39; KCl 0.201; $NaHCO_3$ 1.092; NaH_2PO_4 0.078; $MgSO_4$ 0.123; EDTA 0.073; D(+)-glucose 2.252 and $CaCl_2$ 0.11) and filter sterilized. All preparations, including the remaining sterile buffered enzyme solution for hippocampal dissection were kept in a water bath (37ºC) until they were required.

2.2.2.2 Procedure for primary hippocampal culture

Sprague Dawley rat pups (1-2 days old) were sacrificed via cervical dislocation and decapitated in agreement with the UK Home Office Schedule 1 guidelines under the authority of the UK Animals (Scientific Procedures) Act, 1986. The brain was isolated, the hippocampi dissected out and immediately placed into sterile buffered enzyme solution containing 1.5% papain for 20 minutes at 37ºC. The hippocampi were then placed into 1% BSA solution to stop papain digestion and titrated to dissociate the cells using a series of sterile flame-polished glass pipettes of decreasing tip diameter. The cells were pooled and spun down by centrifugation at 2000 rpm for 2 minutes producing a pellet. The supernatant was removed and the cells resuspended using 1ml of hippocampal culture media. Using a haemocytometer, the cells were counted and diluted down to a density of 3 x 10^5 cells/ml and cells

(100µl per coverslip) were seeded onto the poly-L-lysine coated coverslips. The cells were incubated at 37ºC/5% CO_2 for 1 hour before discarding the excess cells by removing the remaining media and then slowly flooding the petri dishes with 2ml of hippocampal culture media. The cultures were kept and maintained in a humidified incubator at 37ºC/5% CO_2 for upto 14 days *in vitro* (DIV) prior to functional studies. Cytosine β-D-arabinofuranoside (Ara-C at 10µM) was added after 5 DIV to prevent further glial cell proliferation.

2.2.3. Primary culture immunocytochemistry

2.2.3.1. Antibodies

Anti-Microtubule-associated protein 2 (MAP2, neuronal somatodendritic marker, 1:1000 dilution), glial fibrillary acidic protein (GFAP, astrocytic marker, 1:500 dilution), anti-Iba-1 (microglial marker, 1:1000 dilution) and anti-04 (oligodendrocyte marker, 1:1 dilution) were used to identify neurons, astrocytes, microglia and oligodendrocytes respectively with anti-TLR3 (TLR3 marker, 1:200 dilution) used to identify the pattern recognition receptor, TLR3. Anti-GluA1 (GluA1 subunit of AMPA receptor marker) was used at a dilution of 1:50.

2.2.3.2. TLR3 cellular localisation in primary hippocampal cultures

Primary hippocampal cultures between 7-10 DIV were washed in phosphate buffered saline (PBS) three times and then fixed in ice-cold 4% paraformaldehyde (PFA) for 10 minutes before being washed again

three times in PBS. The cells were treated with ice-cold 100% methanol for a further 10 minutes and washed again in PBS (3x washes) before permeabilising the cells with 0.01% Triton-X (in PBS). To prevent non-specific binding, blocking buffer (5% foetal bovine serum (FBS) v/v and 1% BSA w/v in PBS) was added to the cells for 1 hour. Primary antibodies were prepared at the appropriate dilution, with the exception of O4 and its secondary antibody which were added prior to fixation and permeabilisation for 20 minutes, in blocking buffer solution to identify the appropriate cellular components. Controls were also performed where no primary antibody was present. 100µl of the diluted antibody was then applied directly to the coverslips and incubated overnight (16-20 hours) in a wet box at 4ºC. Cultures were then washed three times in PBS to remove any unbound primary antibody and the correct species of fluorescently labelled secondary antibodies (Alexa Fluor 488/555 anti-chicken, anti-mouse and anti-rabbit, all 1:200 dilutions) used simultaneously were prepared in blocking solution. Secondary antibodies were added directly to the coverslips for 1 hour in the dark at room temperature. The coverslips were washed in PBS three times and either kept in dishes for a maximum of three weeks containing PBS or mounted onto slides using Vectashield (containing DAPI) and incubated in the dark at 4ºC until epifluorescent images were taken. Image acquisition was performed with an Olympus BX51W1 microscope with a Q-imaging digital camera, images were taken using WinFluor v3.4.4 imaging software (J. Dempster, University of Strathclyde, Glasgow, UK) and viewed in PicViewer. Cells were imaged using either a 20x/40x/60x water immersion lens with excitation using appropriate filters to visualise the secondary antibody signals. Confocal imaging was also carried out on cells to examine the subcellular localisation of TLR3.

Cells were imaged using either a 20x/40x/60x oil immersion lens on a Leica SP5 TCS confocal system with excitation using appropriate filters to visualise the signals from secondary antibodies using the Leica image capturing software program, LASAF.

2.2.3.3. TLR3 cellular localisation in iPSCs

Primary fibroblast lines were generated and reprogrammed into iPSCs as described previously (Shi *et al.*, 2012; Sposito *et al.*, 2015). The iPSCs were then differentiated into cortical neurons by Dr Graham Robertson. Briefly, once the iPSCs were 100% confluent, the media was replaced by neural induction media (1:1 of N2 consisting of 1 x N-2, DMEM/F-12 GlutaMAX, , 5 μg/ml of insulin, 1mM l-glutamine, 100 μM nonessential amino acids, 100 M2-mercaptoethanol, 50 units/ml of penicillin and 50 mg/ml streptomycin and B27 media consisting of 1 x B27, Neurobasal, 200mM l-glutamine, 50 units/ml penicillin and 50mg/ml streptomycin supplemented with the SMAD inhibitors, dorsomorphin (1μM) and SB431452 (10μM). For 12 days, media was changed daily and then replated onto laminin-coated plates. Cells were fed every 2 days using neural maintenance media as described, replated around day 28 when neurogenesis had occurred to a substantial amount and the replated onto poly-ornithine and laminin-coated plates at day 35 until required for immunocytochemistry (Sposito *et al.*, 2015). The procedure for this experiment was conducted as described in section 2.2.3.2.

2.2.3.4. TLR3 activation and AMPA internalisation

Control primary hippocampal cultures and TLR3 activated (poly I:C, 25µg/ml, 1 hour) between 10-12 DIV were washed in phosphate buffered saline (PBS) three times. Primary antibodies were prepared at the appropriate dilution and 100µl was applied directly to coverslips in a wet box at room temperature for 40 minutes. For this study, GluA1 antibody was required for the staining on AMPARs on the surface of cells and intracellularly showing internalisation of the receptor. Therefore cultures were either not permeabilised (control) or permeabilised for this experiment. For cultures that would not be permeabilised, MAP-2 (neuronal marker) and GluA1 antibodies (AMPA receptor subunit marker) were added and for those being permeabilised, only the GluA1 antibody was added. The coverslips were washed in PBS a further 4 times and then fixed in ice-cold 4% paraformaldehyde (PFA) for 10 minutes before being washed again three times in PBS. To prevent non-specific binding, blocking buffer (5% FBS v/v and 1% BSA w/v in PBS) was added to the coverslips for 30 minutes at room temperature before being washed 3 times in PBS. Correct species of fluorescently labelled secondary antibodies (Alexa Fluor 488/555 anti-chicken and anti-sheep, 1:200 dilution) used simultaneously were prepared in blocking solution. 100µl of secondary antibodies were added directly to the coverslips for 1 hour at room temperature in the dark before being washed again in PBS (4x). 4% PFA was then applied for 10 minutes before the coverslips were washed 3 times and for those coverslips that were not being permeabilised, were dried and mounted onto slides using Vectashield and incubated for 4ºC until epifluorecent images were taken.

For permeabilisation, 0.1% Triton X (in blocking buffer) was added directly to the coverslips for 10 minutes and washed 3 times in PBS. Primary antibody, MAP-2, was prepared at the appropriate dilution and 100µl was applied to the coverslips overnight in a wet box at 4ºC. Coverslips were washed 3 times in PBS and then blocking buffer was added for 30 minutes. Coverslips were then washed again a further 3 times before the secondary antibody of the correct species and dilution was added (100µl) directly to the coverslips for 1 hour, in the dark at room temperature. Again, coverslips were washed 3 times before being dried and mounted onto coverslips and stored at 4ºC until viewed using epifluorescent microscopy.

2.2.3.5. Analysis

Image acquisition was carried out with an OLYMPUS BX51W1 microscope with a Q-imaging digital camera and using WinFlour v3.4.4 imaging software (J Dempster, University of Strathclyde). Cells were imaged using a 20x/40x water immersion lens as appropriate with excitation using appropriate filters to visualize AlexaFlour 488 and 555 secondary antibody signals respectively. Analysis for experiments conducted in section 2.2.3.2 was carried out by taking images and creating Z-stacks from a random cell to determine the cellular localisation of TLR3 within the different types of brain cells. Analysis for experiments conducted in section 2.2.3 was carried out by randomly taking images and determining if AMPA internalisation occurred, fluorescence intensity from 5 neurites per coverslip for naïve and treated cultures was carried out using ImageJ software (NIH, Maryland). For

neurites, n represents the number of neurites from at least three separate cultures. 50 µm rectangular shaped regions of interest were drawn and added to 5 randomly selected neurites per coverslip and all data are expressed as mean ± S.E.M. Data were compared by unpaired t-test with $P < 0.05$ considered significant.

2.2.4. Electrophysiology in neurones of a primary hippocampal culture

2.2.4.1. Whole cell patch clamp electrophysiology recording procedure

The set-up for allowing whole cell patch clamp recording consisted of a faraday cage preventing interference from external electrical noise. Within the cage, a submerged recording chamber with an inverted microscope (Nikon Eclipse TS100, Japan) and manipulator (MP-365 Sutter instrument company, USA) were mounted onto an anti-vibration table (TMC, MA). Cells were perfused (1-2 ml/min) continuously with a HEPES-buffered saline (HBS) containing (in mM): NaCl 140, KCl 5, MgCl2 2, HEPES 10, D-glucose 10 and CaCl2 2. The pH was adjusted to 7.4 ± 0.2 and the osmolarity was corrected to 310mOsm ± 0.2 if required with sucrose. Data was captured using an Axopatch-200B amplifier (Molecular Devices, Sunnyvale, CA, USA) with data digitized (Axon digidata 1322A, CA, USA) at either 4kHz or 10 kHz and filtered at 2 kHz (Fig 2.1). Borosilicate glass micropipettes were pulled using an electrode puller (DMZ-Universal, Germany) providing a resistance range between 4-6 Ohm. Current clamp experiments were carried out using whole cell patch clamp in current clamp mode and IV current

experiments were carried out in voltage clamp mode with glass pipettes filled with an internal solution containing (in mM): $KMeSO_3$ 130, KCl 20, HEPES 10, EGTA 0.5, MgATP 4 and GTP 0.3 with the pH adjusted to 7.2 and the osmolarity corrected to 290-300mOsm using sucrose if required. Experiments were carried out on cultured neurons between 10-14 DIV. Any neurons with an initial resting membrane potential of >-55mV, either naïve or treated neurons, were rejected. Glass electrodes were moved by the manipulator towards the chosen cell until the tip of the electrode touched the cell membrane (which is seen by the increase in resistance, Fig 2.2). At this point, a gentle negative pressure was applied to obtain a seal of high resistance (GΩ seal). Once the gigaseal was obtained, the cell was held at -60mV and short bursts of suction was applied to rupture the membrane, once cell access was obtained, it was left to dialyse and equilibrate with the internal solution for 5 minutes before recording. Current clamp experiments on the spontaneous action potential firing (sAPs) of neurons had a stable 5 minute baseline period before applying the required treatments either acutely or chronically. The experiments were performed with a sampling frequency of 4kHz and a holding potential of -65mV. Voltage clamp experiments to analyse sodium and potassium currents were carried out on acute and chronic poly I:C treated cultures. The sampling frequency was 10kHz with 14 depolarisation steps (-90mV to +40mV) every 20 seconds in which the initial holding current was -70mV. Studies on miniature excitatory postsynaptic currents (mEPSCs) were carried out to determine the effect on quantal neurotransmitter release using controls and TLR3 activated cultures. A 5 minute recording baseline period of spontaneous excitatory postsynaptic currents (spEPSCs) was obtained before two further recordings of 5 minutes in the presence of TTX (0.5µM) was carried out.

This experiment was held at -70mV with a sampling frequency of 10kHz.

2.2.4.2. Acute drug application

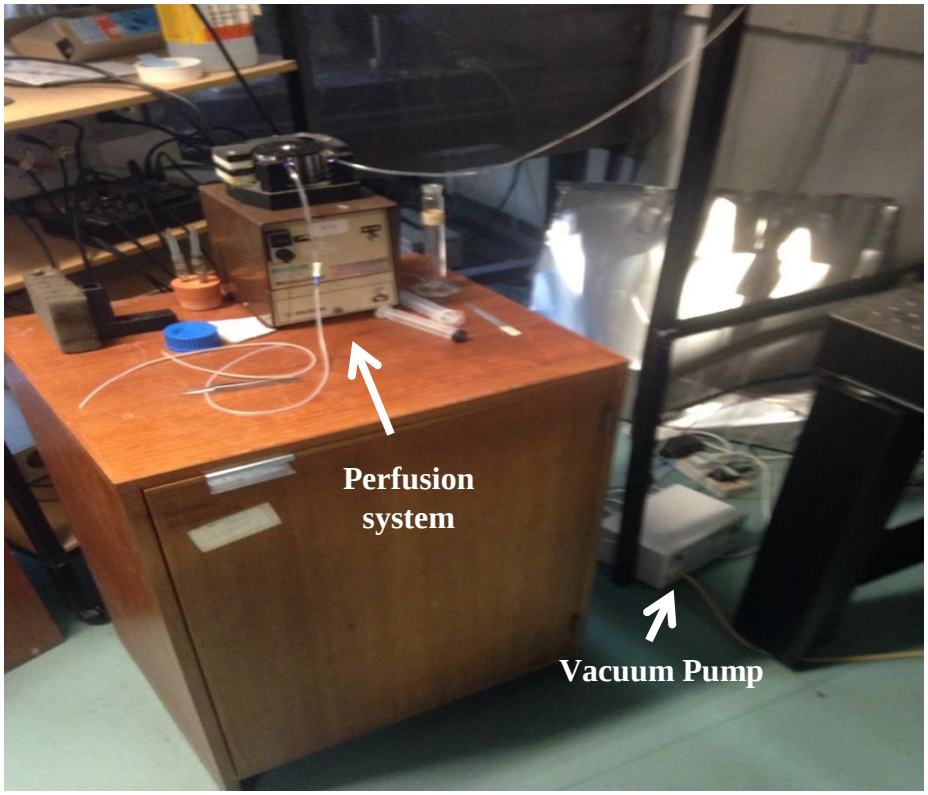
After allowing a 5 minute recording for the baseline to stabilise, drugs were added via the perfusate for 5 minutes and poly I:C treated applications were washed out again via the perfusate for at least 5 minutes. Acutely applied treatments consisted of NBQX/DL-AP5 (AMPA/NMDA receptor antagonists), TTX (sodium channel blocker) and poly I:C at various concentrations (TLR3 activator).

2.2.4.3. Chronic drug application

For chronic application of compounds, drugs were added directly to the hippocampal cultures for 1 hour before recording a 5 minute period following a 5 minute baseline period. Chronically applied drugs consisted of various concentrations of poly I:C, poly A:U (alternative TLR3 activator) and LPS (TLR2/4 activator). For experiments examining the recovery of activity from chronic poly I:C applications, cultures were treated chronically as outlined above, then returned to fresh conditioned media for 3 hours before current clamp recordings were obtained.

2.2.4.4. Analysis

Data were analysed offline using Clampfit, for sodium and potassium current and MiniAnalysis software, for spontaneous AP firing and mEPSCs (Synaptosoft, USA) where n represents the number of cells recorded from a minimum of 3 separate cultures. All data are expressed as mean ± S.E.M.



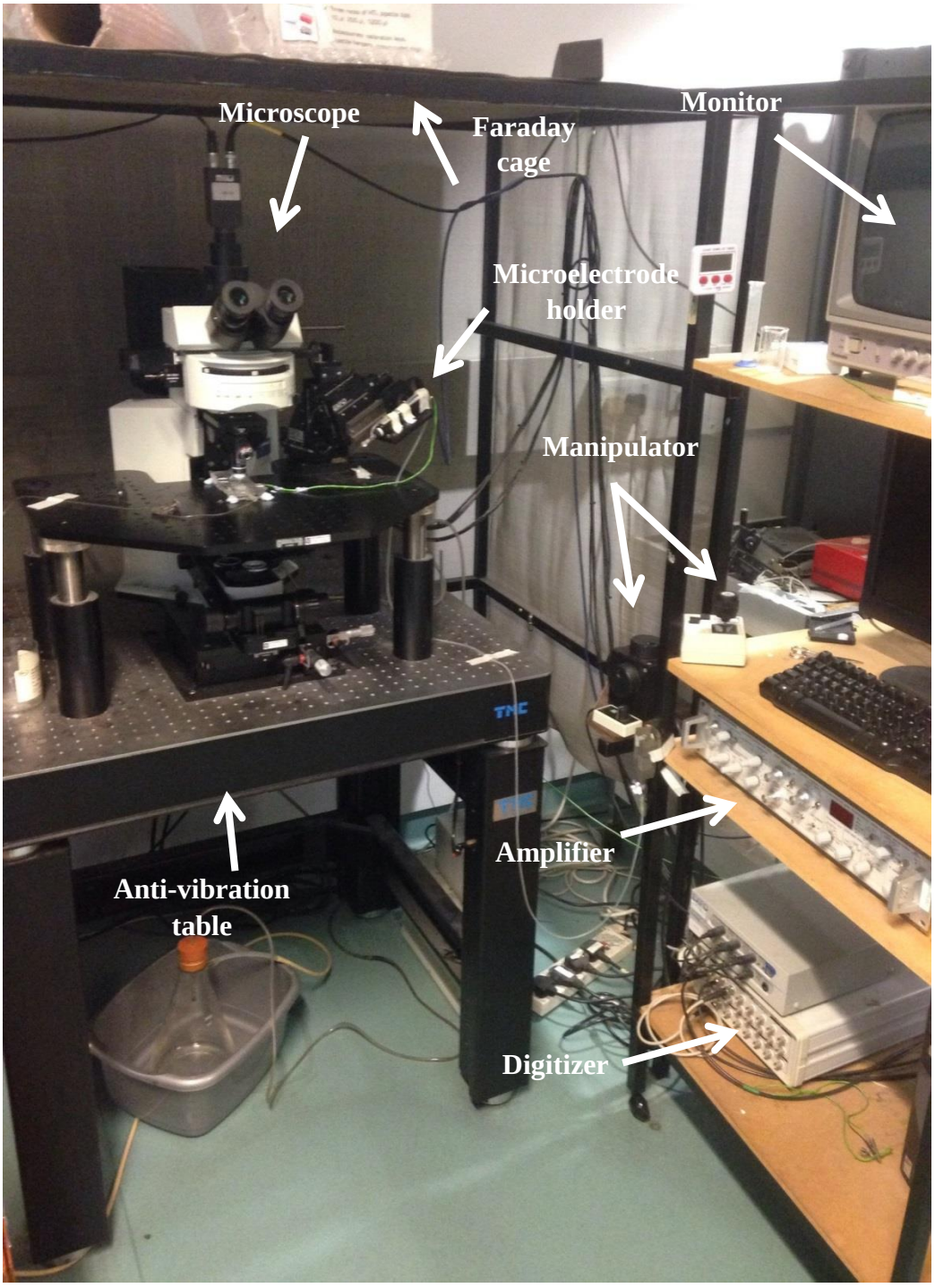


Figure 2.1: Images showing the set-up used in patch clamp electrophysiology.

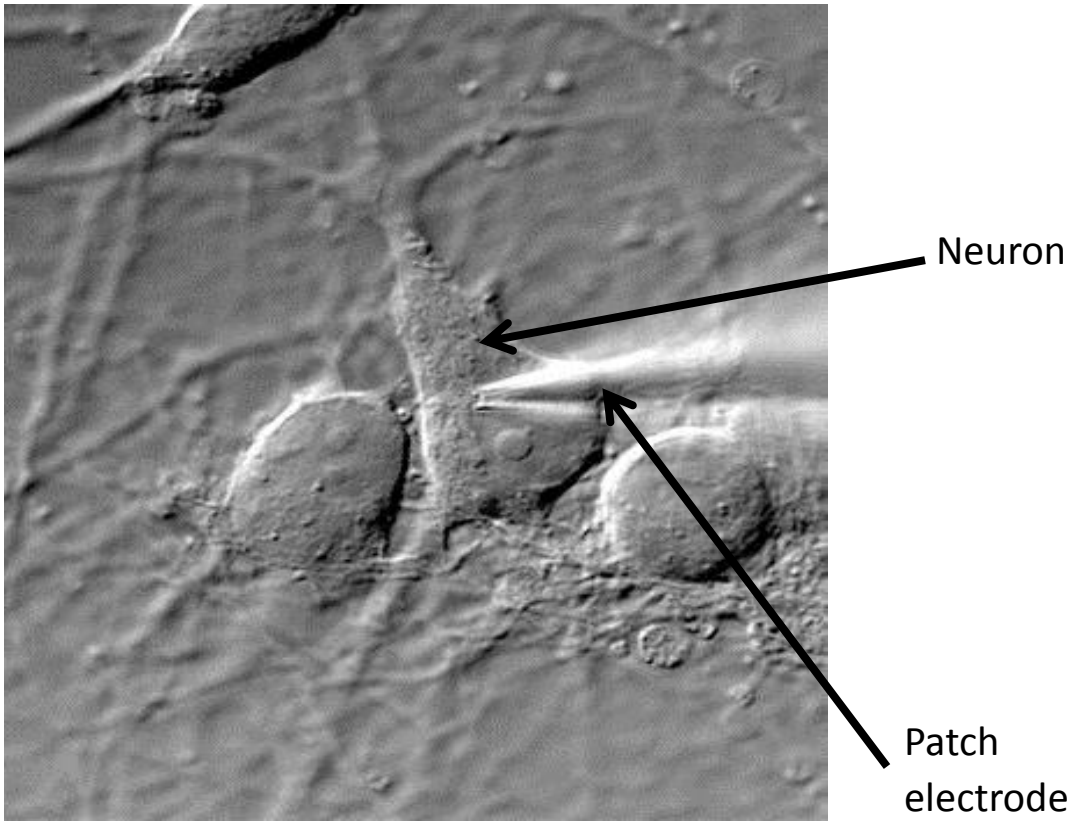


Figure 2.2: Bright field image showing an electrode patching a neuron for whole cell patch clamp electrophysiology.

2.2.5. Transfection of neurons

2.2.5.1. TLR3 subcloning into KKD vector

GFP and the mutant TLR3 A795P (Verstak *et al.*, 2013) were prepared by Dr Rothwell Tate (University of Strathclyde, UK).

2.2.5.2. Transfection procedure

Cultures (8-10 DIV) were transferred into 4 well plates containing 200µl of conditioned media and 200µl of fresh, filter sterilized hippocampal culture media. The coverslips were incubated in a humidified incubator at 37ºC/5% CO_2 until required. GFP and mutant TLR3 transfected cultured consisted of 1µg/ml of the plasmid containing either green fluorescent protein (GFP) or 0.5µg/ml GFP and 0.5µg A795P respectively. This was diluted in 50µl of minimum essential medium (MEM) and 1µl of lipofectamine 2000 diluted in 50µl of MEM for 5 minutes. Both of these preparations were then mixed together for a further 29 minutes before applying 100µl to each coverslip giving a final volume of 500µl and incubated at 37ºC/5% CO_2 for 3 hours. 400µl of the solution was removed and a further 400µl was added to the coverslips (200µl of conditioned media and 200µl of fresh hippocampal culture media) before being maintained in a humidified incubator at 37ºC/5% CO_2 for 2 days until functional experiments are carried out.

2.2.5.3. Whole cell patch clamp electrophysiology on transfected neurons

The procedure for this experiment was conducted as described in section 2.2.4.1. GFP and mutant TLR3 (A7905P) were overexpressed in the culture along with GFP alone allowing identification of transfected neurones. Recordings for sodium and potassium currents and mEPSCs were carried out, as conducted in section 2.2.4.1, on non-transfected cells

and GFP transfected cells overexpressing the appropriate versions of TLR3 in naïve and chronic poly I:C treated cultures.

2.2.5.4. Analysis

Data were analysed as outlined in section 2.2.4.4.

2.2.6. Statistical analysis

All data are expressed as mean ± SEM. Data were compared by unpaired Student's t-tests, one-way analysis of variance with Dunnett's or Tukey's comparison or multiple analysis of variance with Dunnet's comparison as appropriate. Differences were considered significant when $P < 0.05$.

3. DETERMINING THE CONSEQUENCE OF TLR3 ACTIVATION ON SYNAPTIC ACTIVITY IN PRIMARY HIPPOCAMPAL CULTURES

3.1. INTRODUCTION

The CNS was originally thought of as an immune privileged site, however we now know there is an intricate interplay between the CNS and the immune system (Olivieri *et al.,* 2013). Although studies have shown that TLR3 is expressed throughout the brain, our understanding of the consequence of TLR3 activation in the CNS is still very limited (Suh *et al.,* 2009; Wang *et al.,* 2009; Carty and Bowie, 2011). Infections causing TLR3 activation have been shown to induce sickness-like behaviour as a result of proinflammatory changes in the CNS (Engelhardt and Coisne, 2011; Ransohoff and Brown, 2012). Research has shown that these behavioural changes disrupt brain function and in agreement, one study showed behavioural changes seen during infection can not only affect the host directly, but can also affect an unborn foetus during maternal infection (Depino, 2006; Smith *et al.*, 2007). Maternal viral infection during pregnancy is associated with increased incidence of psychiatric and neurodevelopmental disorders including schizophrenia and autism (Patterson, 2011; Arrode-Brusés and Brusés, 2012). Furthermore, TLR3 activation has been shown to inhibit memory retention, adult neurogenesis, neural plasticity, hippocampal dependent learning and AMPA receptor expression in the hippocampus (Cameron *et al.,* 2007; Okun *et al*., 2011). Moreover, a recent study showed that

excitatory synaptic transmission in spinal cord slices were impaired in TLR3 knockout-mice demonstrating a role of TLR3 in the regulation of synaptic transmission (Liu *et al.*, 2012). In contrast, another study by Okun and colleagues showed that increased levels of the AMPA receptor subunit GluA1 in the CA1 region of the hippocampus in TLR3 knockout-mice demonstrating undescribed roles of TLR3 activation being a suppressor of synaptic plasticity and synaptic transmission (Okun *et al*., 2010). These studies reveal opposite effects on synaptic transmission in the absence of TLR3, hence examining the consequence of TLR3 activation on spontaneous AP firing may help elucidate the exact role played by TLR3.

Primary hippocampal cultures are a well-established method for investigating the modulation of neuronal function due to its ease in visualisation and electrophysiological recording. There are many benefits of using this system including the ease of patch clamp recording and pharmacological manipulation. Therefore, due to the benefits and ease of using this system, I decided to carry out my initial investigation on this system to determine TLR3 expression and determine the role of TLR3 activation on the modulation of hippocampal function.

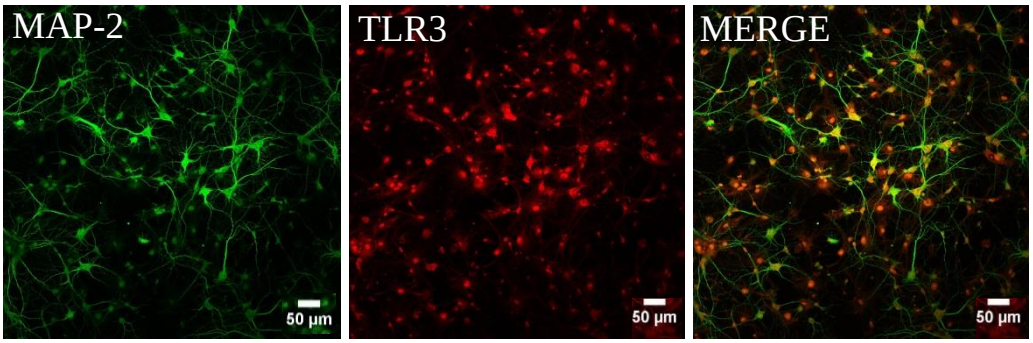
The specific experimental aims described in this chapter were to assess the expression and subcellular localisation of TLR3 on cells within the cultures and to determine the consequence of TLR3 activation on hippocampal synaptic activity. All experiments were performed using immunocytochemistry and whole cell patch clamp electrophysiology in current clamp mode (see sections 2.2.3 and 2.2.4).

3.2. RESULTS

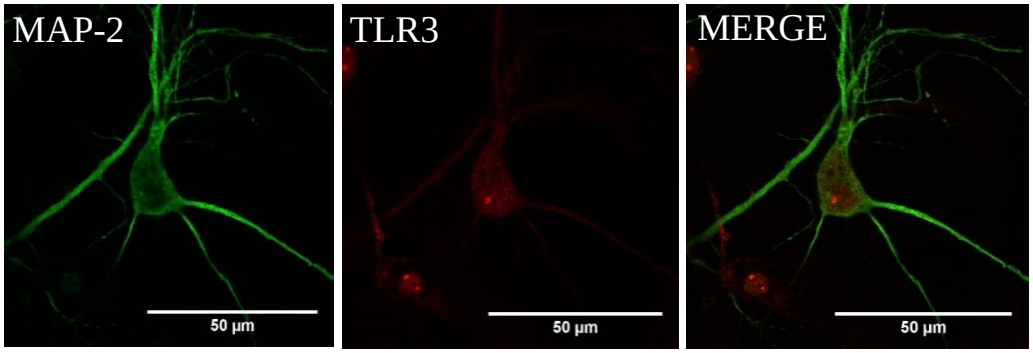
3.2.1 Neurons, astrocytes and oligodendrocytes express TLR3 within primary hippocampal cultures

Immunocytochemical staining was performed to examine TLR3 expression and determine the cellular localisation of TLR3 in primary rat hippocampal cultures. Epifluorescent microscopy revealed that TLR3 was present in neurons (Fig 3.1A), astrocytes (Fig 3.1C) and oligodendrocytes (Fig 3.1D). Z-stacks using single planes confirmed the subcellular localisation in neurons to be intracellular only (Fig 3.1B). Microglia was also found within the cultures however staining for TLR3 was not confirmed due to the primary antibodies being of the same species and issues with availability of antibodies in different species.

A



B



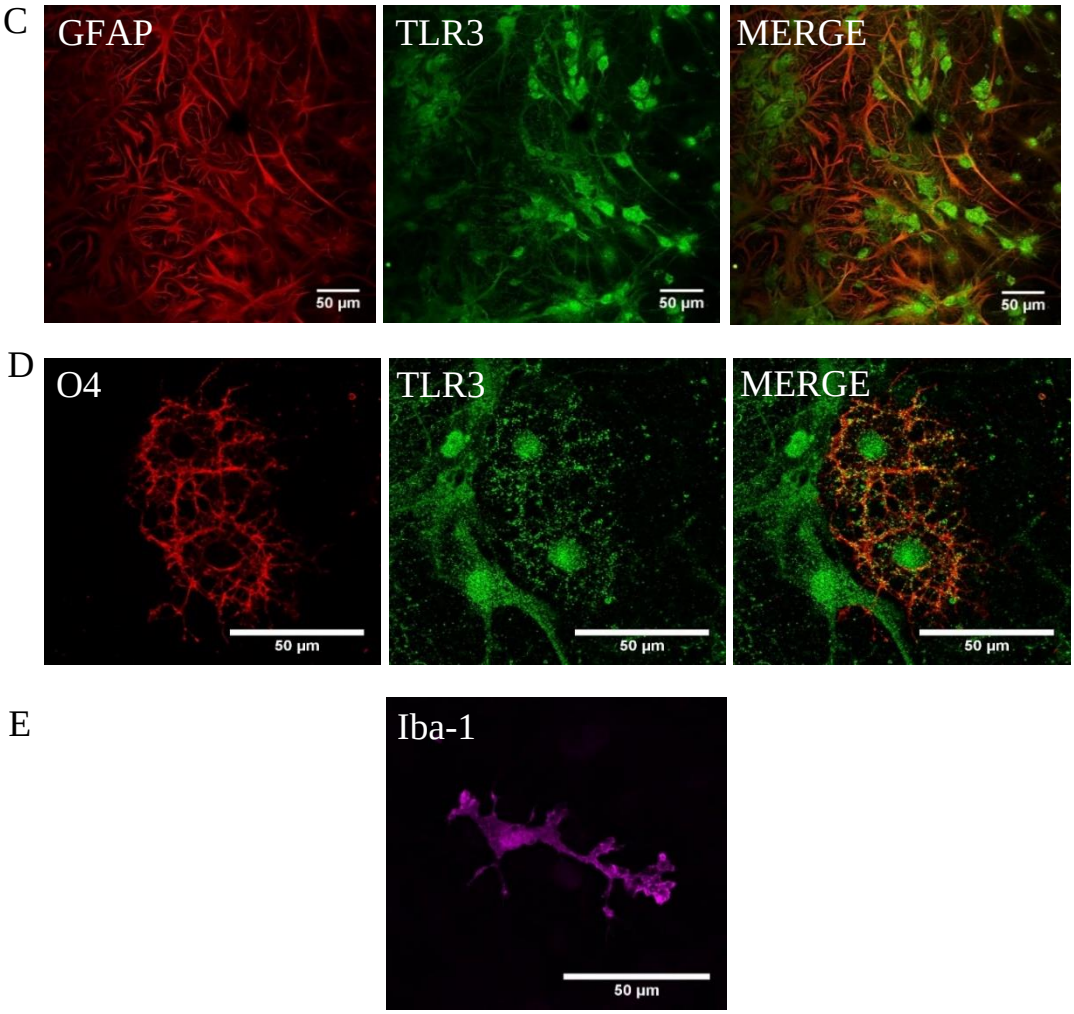


Figure 3.1: TLR3 is expressed on neurons, astrocytes and oligodendrocytes in primary hippocampal cultures.

Representative confocal images showing A) neurons (green) and TLR3 (red) and a merged image (yellow) revealing neuronal TLR3 expression, B) neurons (green) and TLR3 (red) and a merged image (yellow) revealing subcellular localisation is intracellular C) Astrocytes (red), TLR3 (green) and merged (yellow) revealing astrocytic TLR3 localisation D) Oligodendrocytes (red), TLR3 (green) and merged (yellow) revealing TLR3 is present in oligodendrocytes, E) small numbers of microglia (magenta) are present in primary hippocampal cultures.

3.2.2. Human neurons express TLR3

TLR3 has been shown to be present in cultured human neurons and human brain tissue (Préhaud *et al.*, 2005; Jackson *et al.*, 2006). Therefore, I obtained induced pluripotent stem cells (iPSCs)-derived neurons from healthy subjects and carried out immunocytochemistry to determine the presence of TLR3. TLR3 was found to be expressed on the surface and intracellularly of neurons and further expression was found on cells not stained with MAP2 which are presumed to be neural progenitor cells (Fig 3.2).

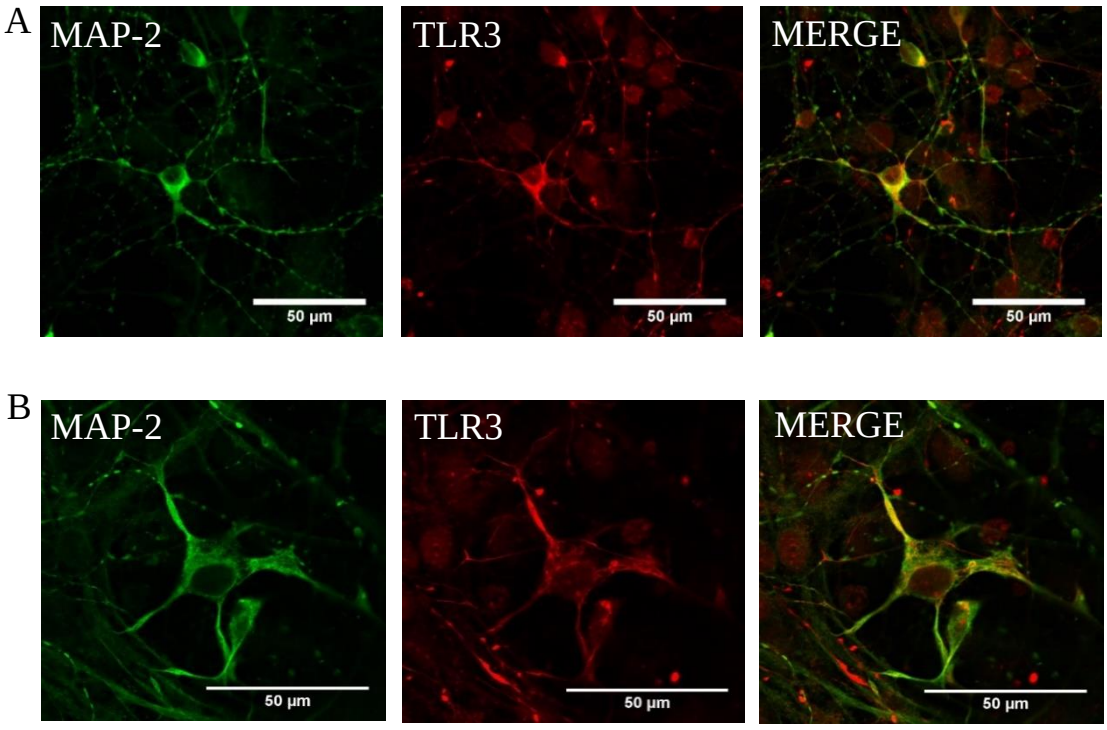


Figure 3.2: TLR3 is expressed on human neurons.
Representative confocal images showing A) neurons (green), TLR3 (red) and a merged image (yellow) revealed TLR3 expression in iPSC-derived human neurons and B) neurons (green) and TLR3 (red) and a merged image (yellow) revealing subcellular localisation is intracellular.

3.2.3. TTX and NBQX/DL-AP5 application abolishes spontaneous AP firing in primary hippocampal cultures

Having established that TLR3 is expressed on the cell types in the primary hippocampal culture, I went on to investigate the effect of TLR3 activation on the modulation of hippocampal activity through monitoring spontaneous action potential (AP) firing. However, before carrying out these experiments, confirmation that spontaneous AP firing within the cultures are sodium channel dependent and synaptically driven was required. Application of TTX (0.5µM) significantly reduced the spontaneous AP firing (0Hz, n=4, $P<0.001$) in comparison to naïve cells (123 ± 27Hz, n=4). In addition, application of NBQX (20µM) and DL-AP5 (100µM), AMPA and NMDA receptor antagonists respectively, also abolished spontaneous AP firing (0Hz, n=3, $P<0.001$) in comparison to the control (Fig 3.3D).

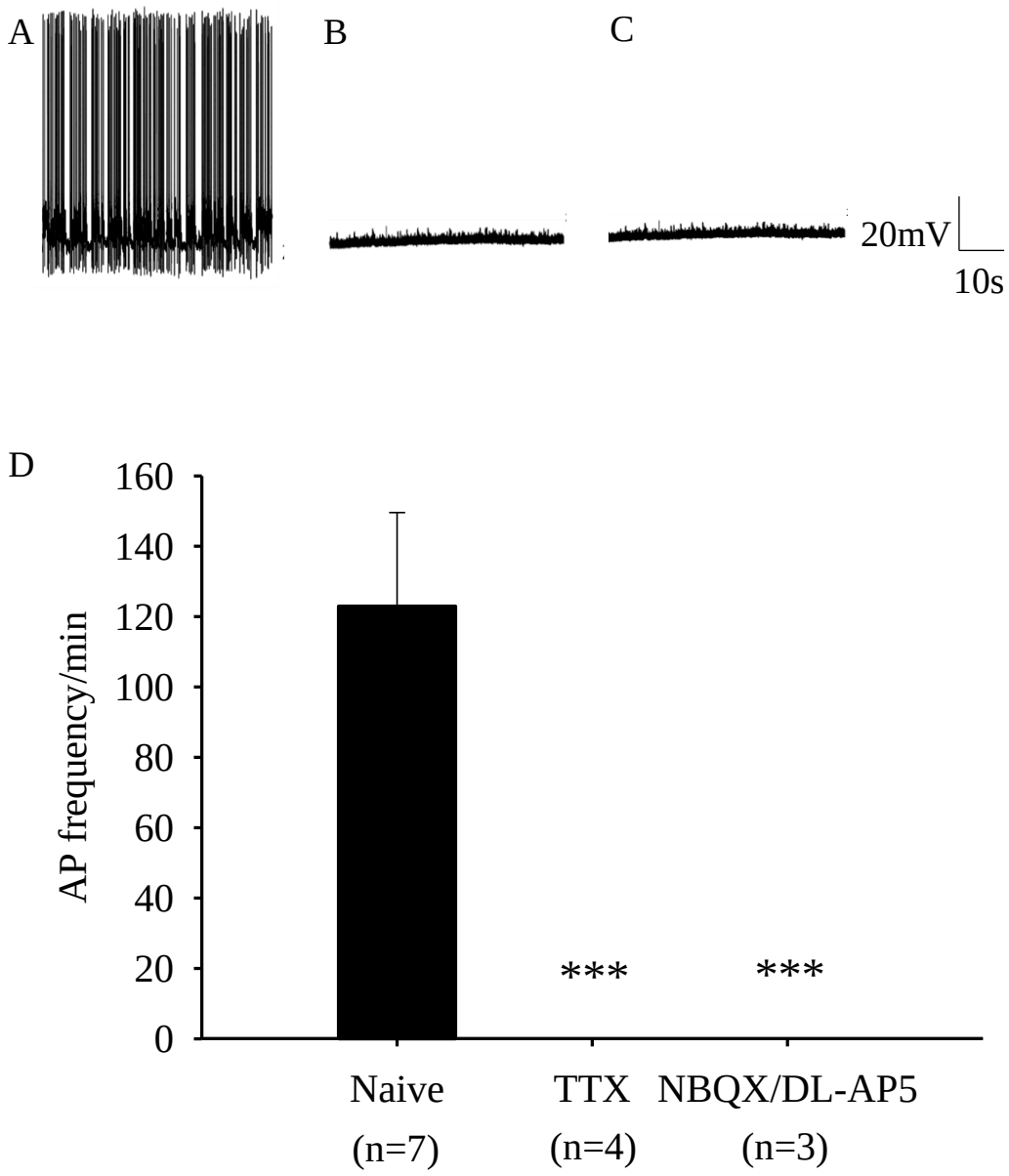


Figure 3.3: TTX and NBQX/DL-AP5 application confirms spontaneous AP firing is sodium channel dependent and synaptically driven.

Representative traces of AP firing exposed to A) control, B) TTX and C) NBQX/DL-AP5. D) Bar chart illustrating TTX and NBQX/DL-AP5 both abolish AP firing, ***P < 0.001 using a one way ANOVA with Dunnett's post hoc test. Data are mean ± S.E.M, taken from at least 3 different cultures.

3.2.4. Short-term TLR3 activation reduces spontaneous AP firing

Having confirmed that spontaneous AP firing within my cultures is sodium channel dependent and synaptically driven, I then investigated whether TLR3 activation modulates spontaneous AP firing. Initially, I examined the effect of short-term (5min) TLR3 activation on hippocampal synaptic activity. Application of poly I:C (1µg/ml) resulted in an initial increase in spontaneous AP firing before significantly reducing AP firing (45.7 ± 11.6% of control, n=7, $P<0.01$) with activity fully recovering upon washout (102.8 ± 8.2% of control, n=7). The effects of poly I:C were concentration-dependent as poly I:C (25µg/ml) resulted in a significant decrease in synaptic activity (82.3 ± 5.6% of control, n=6, $P<0.05$) which fully recovered upon washout (106.6 ± 15.4% of control, n=6). Furthermore, poly I:C (200µg) resulted in a greater reduction of synaptic activity (58.1 ± 8.4% of control, n=6, $P<0.01$) with little recovery (68.3 ± 8.1% of control, n=6). In addition, control experiments were carried out using the vehicle (water) which resulted in a non-significant effect confirming that the reduction in activity was due to the action of poly I:C (Fig 3.3).

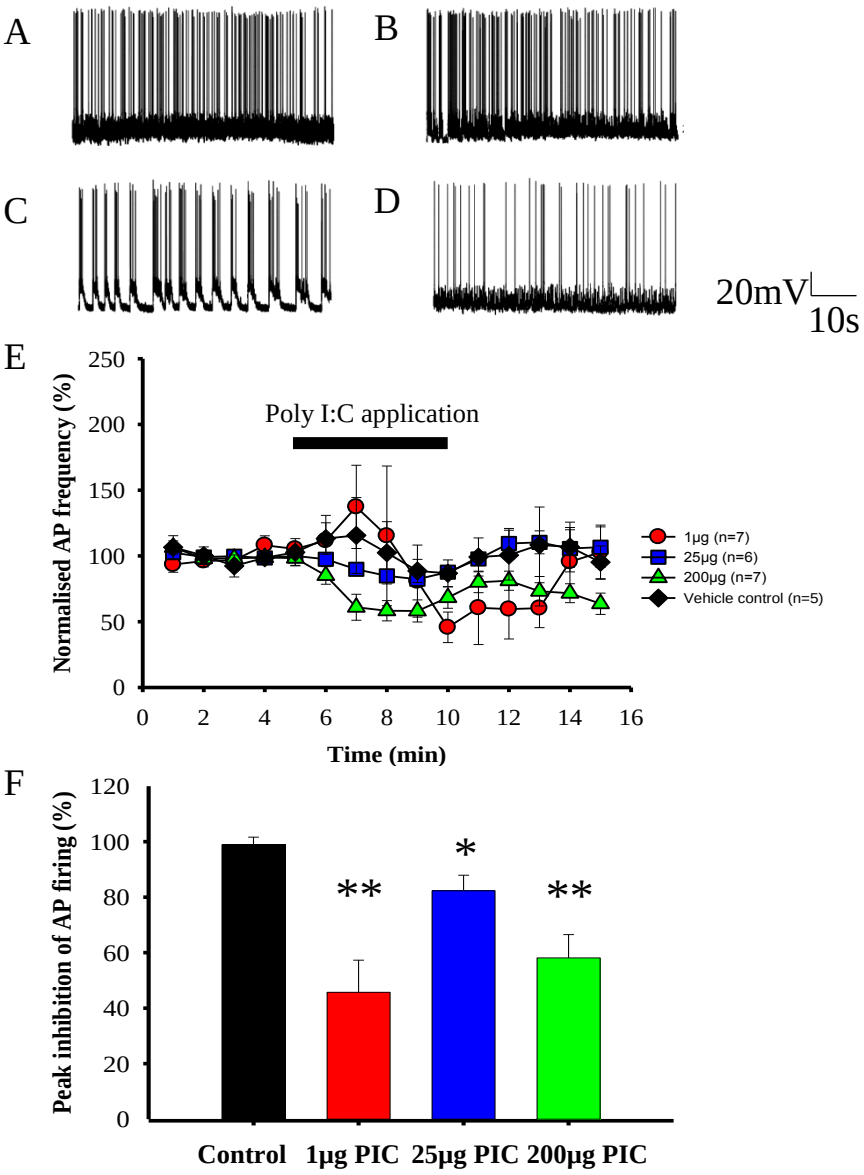


Figure 3.4: Short-term poly I:C application reduces spontaneous AP firing.

Representative traces of AP firing from neurons exposed to A) vehicle, B) poly I:C (1µg/ml), C) poly I:C (25µg/ml) and D) poly I:C (200µg/ml). E) Time course revealing concentration-dependent and reversible effects of poly I:C. F) Bar chart summarising the maximum effect of poly I:C in spontaneous AP firing, *P< 0.05, **P < 0.01 using a one way ANOVA with Dunnett's post hoc test. Data are mean ± S.E.M, taken from at least 3 different cultures.

3.2.5. Long-term TLR3 activation reduces spontaneous AP firing

After establishing that short-term poly I:C application resulted in a significant reduction in spontaneous AP firing, experiments were carried out to determine the consequence of poly I:C application over a longer period (1h). Similar to short-term poly I:C application, long-term poly I:C application resulted in a significant reduction in spontaneous AP firing in a concentration-dependent manner. Application of poly I:C (1µg/ml) resulted in a significant reduction of spontaneous firing (67.3 ± 3.2 AP/min, n=6, $P<0.01$) in comparison to vehicle (163.4 ± 15.9 AP/min, n=17). Furthermore, poly I:C (25µg/ml) led to a further reduction in spontaneous AP firing frequency (1.5 ± 0.8 AP/min, n=8, $P<0.001$) when compared to vehicle (Fig 3.5E) with activity returning (100.5 ± 31 AP/min, n=8, $P<0.01$) following 3 hours recovery in conditioned media (Fig 3.6C). To confirm TLR3 involvement, another agonist of TLR3, poly A:U and an agonist of TLR2/4, LPS, was used. Application of poly A:U, resulted in a significant reduction in spontaneous AP firing (50.2 ± 10 AP/min, n=5, $P<0.01$) however LPS was without effect (Fig 3.7C).

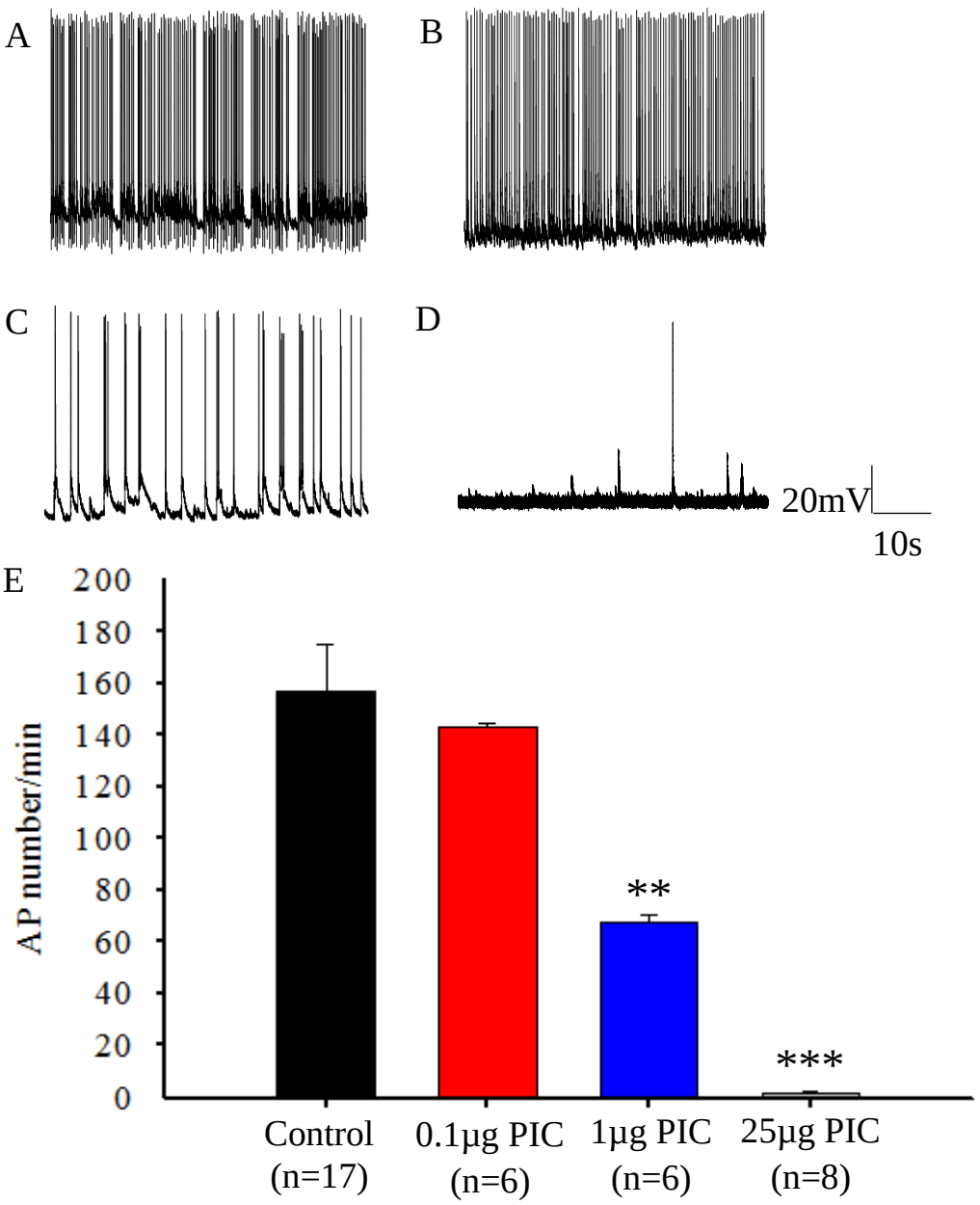


Figure 3.5: Long-term poly I:C application results in a concentration dependent reduction in AP firing.

Representative traces of AP firing from neurons exposed to A) vehicle, B) poly I:C (0.1µg/ml), C) poly I:C (1µg/ml) and D) poly I:C (25µg/ml). E) Bar chart summarising the effect of poly I:C on spontaneous AP firing, **P< 0.01, ***P < 0.001 using one way ANOVA with Dunnett's post hoc test. Data are mean ± S.E.M, taken from at least 3 different cultures.

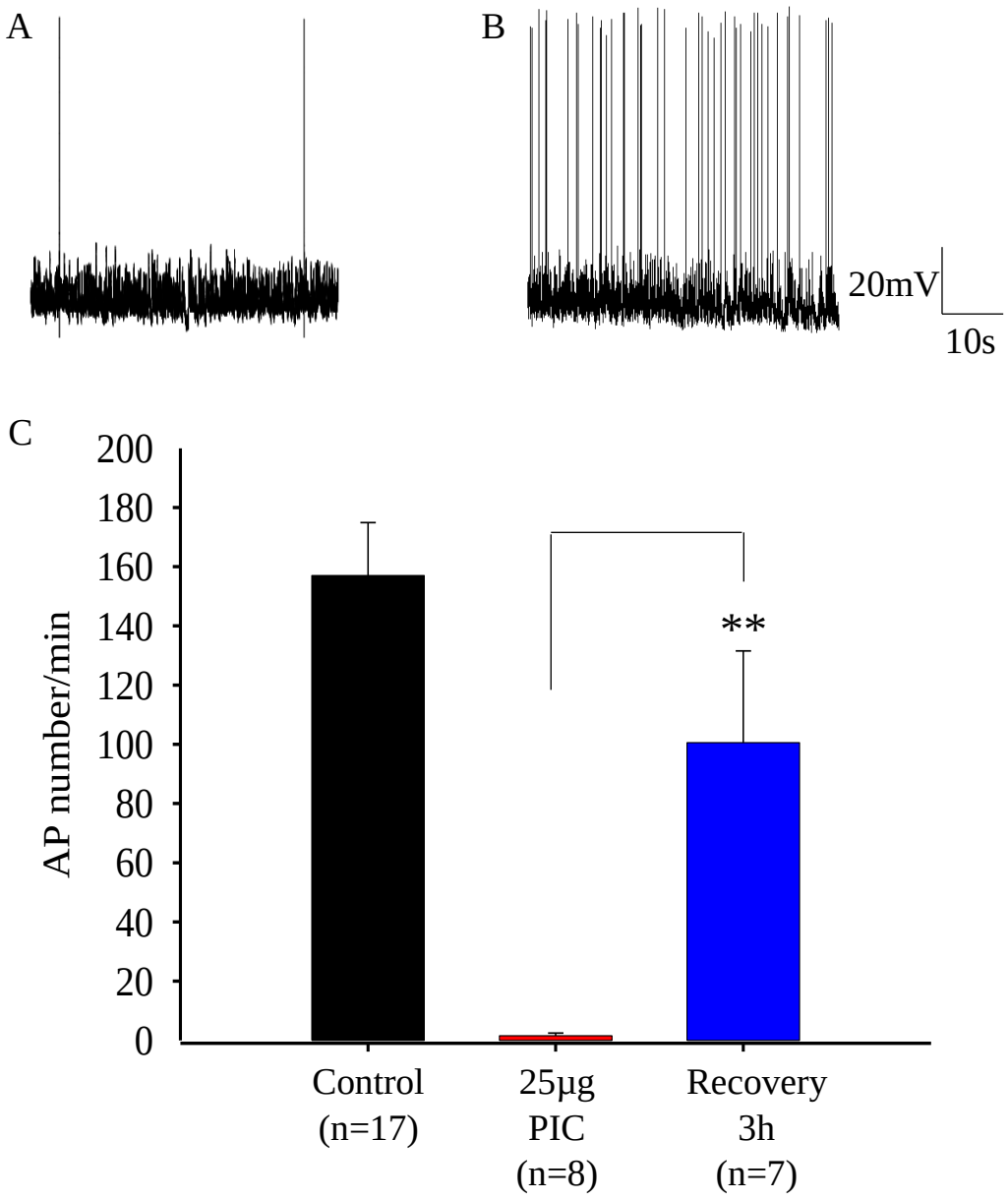


Figure 3.6: Poly I:C induced inhibition of AP firing is reversed upon washout.

Representative traces of AP firing from neurons exposed to A) poly I:C (25µg/ml) and B) recovery (3h). C) Bar chart summarising the effect of poly I:C on spontaneous AP firing is reversible, **P < 0.01 using a one way ANOVA with Tukey's post hoc test . Data are mean ± S.E.M, taken from at least 3 different cultures.

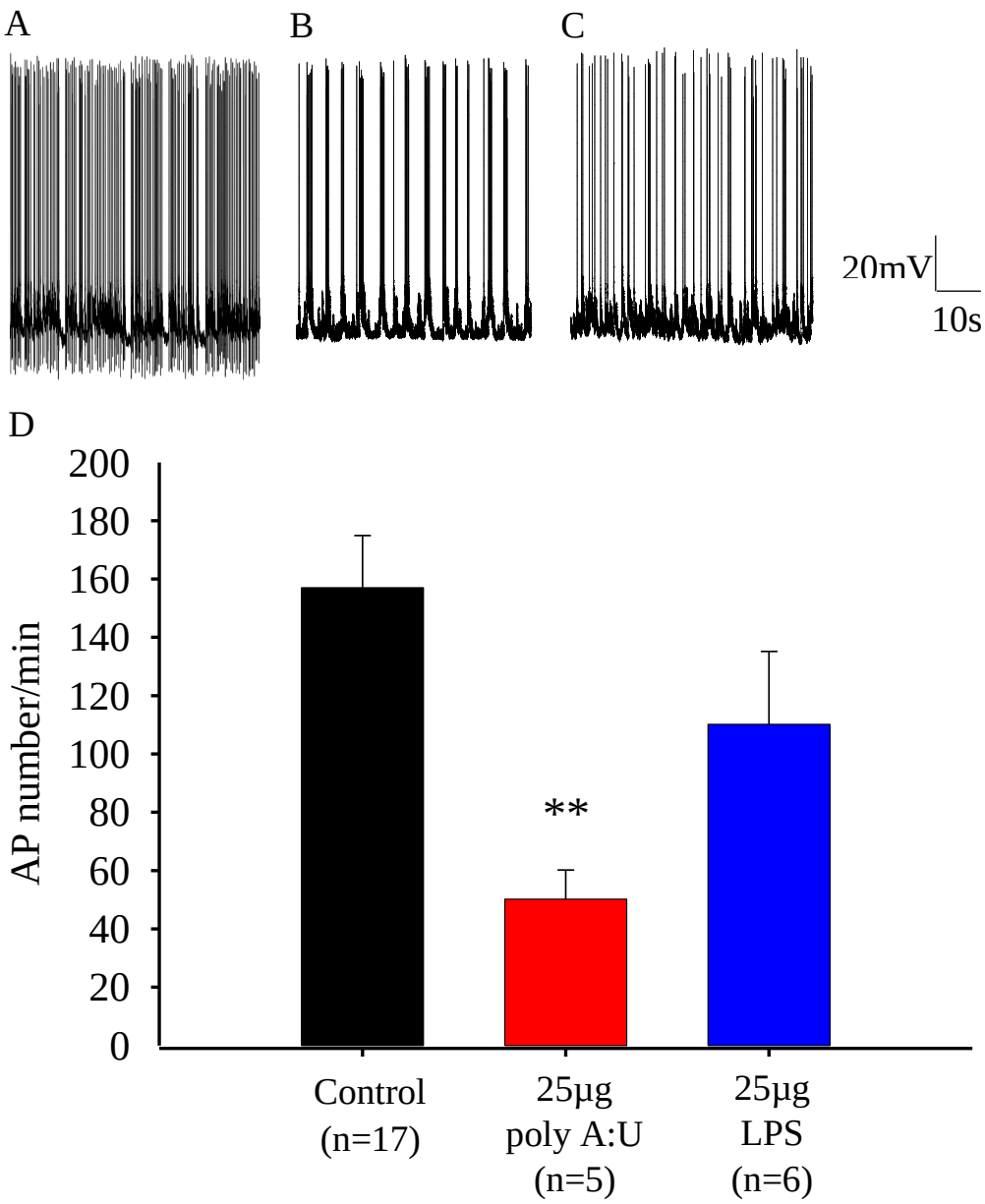


Figure 3.7: Application of poly A:U but not LPS results in a significant reduction in AP firing.

Representative traces of AP firing from neurons exposed to A) vehicle, B) poly A:U (25µg/ml) and C) LPS (25µg/ml). D) Bar chart summarising the effect of poly A:U and LPS on spontaneous AP firing, **P < 0.01 using one way ANOVA with Dunnett's post hoc test. Data are mean ± S.E.M, taken from at least 3 different cultures.

3.2.6. TLR3 activation using a lower concentration of poly I:C with longer exposure time reduces spontaneous action potential firing with recovery upon washout

Having established that applying poly I:C (0.1µg/ml, 1h) resulted in no effect on spontaneous AP firing, experiments were carried out to determine the effect of the lower concentration of poly I:C over a longer exposure time (18h). Application of poly I:C (0.1µg/ml, 18h) resulted in the complete abolition of spontaneous AP firing (0.2 ± 0 AP/min, n=6, $P<0.001$) with no effect on recovery in firing after 3 hours. Furthermore, allowing cells to recover for longer (18h) caused a significant increase in recovery of AP firing (33.4 ± 0.7 AP/min, n=7, $P<0.05$) when compared only to poly I:C application (0.1µg/ml ,18h, Fig 3.8E).

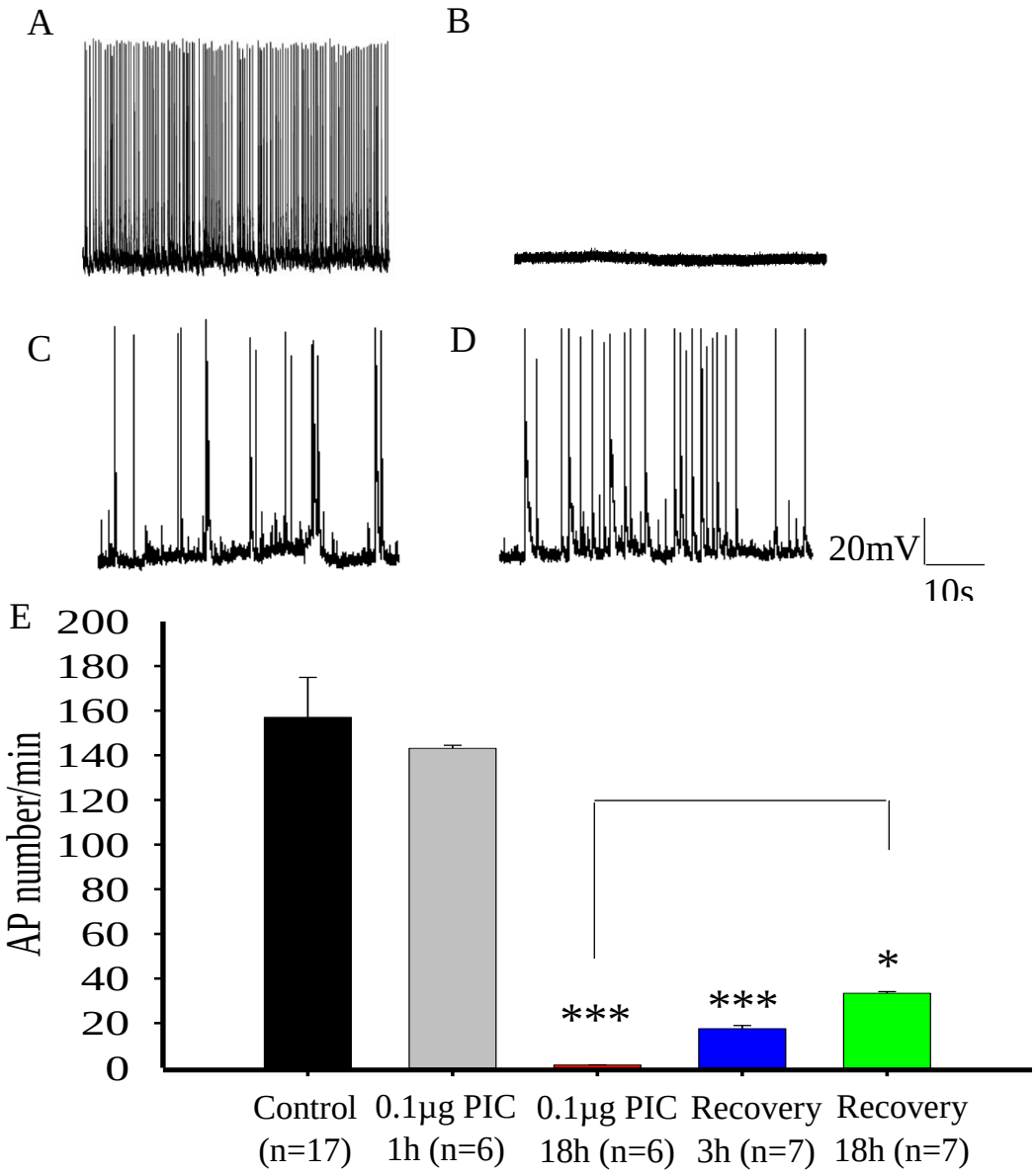


Figure 3.8: Chronic poly I:C application results in significant AP firing reduction and recovery upon washout.

Representative traces of AP firing from neurons exposed to A) vehicle, B) poly I:C (0.1µg/ml) for 18h, C) recovery (1h) and D) recovery (18h). E) Bar chart summarising the effect of poly I:C for longer exposure on spontaneous AP firing is reversible, *P < 0.05, ***P < 0.001 using one way ANOVA with Tukey's post hoc test. Data are mean ± S.E.M, taken from at least 3 different cultures.

3.2.7. TLR3 activation does not affect resting membrane potential

I next investigated whether an effect on the AP firing could be explained by modulation of the resting membrane potential (RMP). Short-term TLR3 activation had no effect on the RMP of neurons during recordings studying AP firing with poly I:C (200µg) resulting in a 0.4 ± 0.2mV difference (n=7). Similarly, long-term TLR3 activation (25µg poly I:C) had no effect on the initial RMP prior to holding at -65mV (control: 58.6 ± 0.6mV, n=17; poly I:C: 58.9 ± 0.8mV, n=8).

3.3. DISCUSSION

In this chapter, I demonstrated that TLR3 is expressed on multiple cell types in primary rat hippocampal cultures and on human neurons that have been differentiated from induced pluripotent stem cells. Furthermore, I show that poly I:C reversibly inhibits AP firing via TLR3 activation.

3.3.1. TLR3 is expressed on cells in primary hippocampal cultures and human neurons

Studies have shown TLR3 to be expressed on every cell type in the CNS (Costello and Lynch, 2013; Hoyo-Becerra *et al.*, 2013). Hence, initial experiments were performed to confirm TLR3 expression in neurons, astrocytes, oligodendrocytes and microglia within primary hippocampal cultures. The results showed TLR3 to be expressed in neurons, astrocytes and oligodendrocytes in the primary hippocampal cultures. Although studies have shown TLR3 expression to exist on microglia, the only CNS cell type with all TLRs expressed, unfortunately due to issues with antibody species, I was unable to show this in my own studies. Both antibodies used for microglia and TLR3 were of the same species and it was difficult finding a suitable antibody to use for immunocytochemistry on rat primary hippocampal cultures to allow this study to be carried out.

I also carried out immunocytochemistry studies on induced pluripotent stem cells (iPSC) derived neurons. These are cells that have been

artificially reprogrammed from adult fibroblasts and can give rise to every cell type, in this case, neurons (Kwon *et al.,* 2012; Breton *et al.,* 2013). They are extremely useful as they propagate indefinitely, serve as a single cell source and have the ability to be patient-matched but more importantly, they are cells taken from humans therefore using them to study diseases and consequence of these diseases better relate to what happens in an individual rather than using animals (Takahashi *et al.,* 2006; Takahashi *et al*., 2007; Wu *et al*., 2011). Immunocytochemistry studies showed expression of TLR3 present intracellularly and on the cell surface. In contrast, the results from the primary cultures showed expression of TLR3 to be only intracellular, which is in agreement with the many studies that provided evidence of TLR3 expression both intracellularly on neurons in murine and human neurons (Préhaud *et al.*, 2006; Trudler, 2010). However, evidence has also suggested expression of TLR3 on the membrane surface of cell types under certain conditions (Liu *et* al., 2012). A reasonable explanation as to why our study showed TLR3 expression on both the cell surface and intracellularly on human neurons but only intracellularly on cultured neurons may be that the human neurons display a different TLR profile and localisation. Furthermore, expression of TLR3 intracellularly on both cultured and human neurons suggests the same pathway used by immune cells for TLR3 signalling, the classical TRIF pathway, may underlie any modulatory effects seen in the present study (Takeda and Akira, 2005). Due to the time limitations, I did not determine the functional consequence of TLR3 activation on hippocampal synaptic activity on human neurons however was able to do so in the primary hippocampal cultures.

3.3.2. Spontaneous action potential firing is sodium channel-dependent and synaptically driven

Confirmation of spontaneous APs being sodium channel-dependent and synaptically driven was shown through their sensitivity to the application of TTX which is a sodium channel blocker and NBQX/DL-AP5 which are ionotropic glutamate receptor antagonists. These were required first before determining the consequence of TLR3 activation on excitatory neurotransmission. The results showed the complete abolition of spontaneous AP firing which is in agreement with previous studies (Aomine, 1988; Lee and Ruben, 2008; Hall et al., 2012). Action potential propagation, neurotransmitter release and activation of receptors provide the means by which neurons communicate and transmit required information through neurotransmission (Pereda, 2014). Spontaneous AP firing relies on a regenerative wave of ion channels opening and closing in the membrane. Depolarisation at the axon hillock to threshold causing the propagation of an action potential resulting in the activation of sodium channels which initiates the regeneration. Therefore as an action potential firing is an “all or none” event, the presence of TTX blocking sodium channels prevents action potentials from generating (Faisal and Laughlin, 2007; Kole and Stuart, 2012). When an action potential reaches the axon terminal, a release of neurotransmitters by exocytosis due to calcium release occurs and diffuses across the synaptic cleft. These neurotransmitters bind to receptors (NMDA/AMPA) on the postsynaptic cell resulting in the opening of ion channels altering the postsynaptic cell membrane potential (Rogawski, 2011; Debanne *et al.*, 2013). Blocking these

receptors using NBQX/DL-AP5 prevents ion channels opening, thus inhibiting the propagation of an action potential. Therefore, this confirms action potential firing is sodium channel-dependent and synaptically driven.

3.3.3 TLR3 activation reversibly inhibits spontaneous action potential firing

It is well known that TLR3 activation induces sickness-like behaviour and depressive-like activity in response to viral infections causing various symptoms including fever, fatigue, coldness and numbness (Dantzer, 2009; Reisinger *et al.,* 2015). However our knowledge regarding TLR3 activation and its consequence on neuronal activity is limited. Therefore I investigated the effects of TLR3 activation on spontaneous AP firing over both short and long periods and whether the effects were reversible.

Short term poly I:C application (25µg/ml) resulted in a significant reduction of AP firing in neurons. In addition, a higher concentration (200µg/ml) and a lower concentration (1µg/ml) were used to determine if there was a concentration dependent effect. The data suggests that not only is TLR3 activation resulting in the reduction of synaptic activity but there is also a concentration dependent effect. In addition, experiments using a vehicle control, which was used to deliver the poly I:C, were carried out and resulted in no change in the synaptic activity suggesting that the reduction in neuronal activity is due to the effects of poly I:C. Experiments using poly I:C from an alternative source was also carried

out to show that the effects seen on the activity of neurons was solely due to poly I:C activating TLR3 and not due to the preparation of the TLR3 activator. This was confirmed by using PIC from Invivogen, as opposed to Sigma which resulted in the abolition of synaptic activity which is in agreement with the poly I:C used in these studies from Sigma. Furthermore, a short-term application of poly I:C resulted in a rapid concentration dependent response on AP firing. In addition, not only was it confirmed that there was a concentration dependent decrease through short-term application but the recovery of the AP firing during the washout stages was also apparent. Therefore, further experiments allowing a longer recovery time (18h) was carried out to help determine if the functional changes occurring in the cell required more time to reverse. Recovery was seen which suggests that the cells are still viable however studies on cell viability would have to be carried out to fully confirm this. Moreover, many studies have previously shown TLR3 activation has no effect on cell viability (Cameron *et al.,* 2007; Nojiri *et al.,* 2013; Patel and Hackam, 2013). TLR3 activation has been shown to be neuroprotective against kainate-induced excitotoxicity in the lab (data not published) therefore it is possible that the reduction in AP firing may be a neuroprotective property of neurons against viral infections. Furthermore, previous studies have shown TLR3 to be neuroprotective. One study showed that TLR3 activation protected the immature brain against ischaemic injury through pre-conditioning by reducing infarct volume (Shi *et al.,* 2013). In agreement, it was shown that ischemia activates astrocytes where the process known as astrogliosis can cause damage and prevent neuronal repair. It was demonstrated that TLR3 activation induced neuroprotection via attenuating astrogliosis and reducing infarct volume which lessened neuronal damage (Li *et al.,*

2015). In response to infection, TLR3 activation can also trigger anti-inflammatory cytokine production, neuroprotective mediators and products that enhanced survival of neurons (Bsibsi *et al.,* 2006). On the other hand, the effect on AP firing may be a disturbance of neuronal functional properties that could be irreversibly damaging to the neuron. It is reasonable to suggest the effect is not irreversible as the data shows significant reversibility of AP firing during both short-term and long-term PIC application and as mentioned previously, studies have shown TLR3 activation doesn't affect cell viability (Cameron *et al.,* 2007; Nojiri *et al.,* 2013; Patel and Hackam, 2013). However, although allowing time for 18 hours application at the lowest concentration PIC showed significant reversibility, it was still significantly lower than the AP firing of non-treated neurons. In the context of viral infections, infected individuals generally feel unwell for a few days to a week and if this effect on AP firing was related to virally-mediated behavioural changes in response to TLR3 activation, there may be multiple mechanisms involved requiring at least a few days recovery (Miranda *et al.,* 2010; Forrest *et al.,* 2012). It may be beneficial to carry out further experiments on recovery on neurons over a few days to determine if neuronal excitability fully recovers. Using LPS, an activator of TLR2/4, resulted in no effect on AP firing suggesting the effect of PIC on hippocampal synaptic activity is TLR3-mediated. However to fully confirm this, agonists for every TLR would be required to be studied to determine this.

The reduction of synaptic activity from both short-term and long-term application was shown to have no effect on resting membrane potential therefore this is not the mechanism of action. However other suggestions

to explain why spontaneous AP firing is reduced may be through action potentials being prevented or inhibited and may be due to many factors such as sodium channel inhibition, inhibitory-excitatory imbalance, AMPA receptors being internalised or blocked, inhibition of neurotransmitter release from vesicles or the release of cytokines. One study has indicated that the effect of TLR3 activation on spontaneous AP firing may be via AMPA receptor internalisation (Okun *et al.,* 2010). The results revealed previously undescribed roles for TLR3 through inhibiting memory retention and modulating CNS plasticity when comparing performances of TLR3 wildtype (WT) and TLR3 knock-out (KO) mice. They showed that memory retention, a requirement for working memory, is regulated by neurogenesis as KO mice, when compared to WT had enhanced neurogenesis and working memory. Furthermore, it is well established that AMPARs play a critical role in learning, memory and synaptic plasticity (Sanderson *et al*., 2008). The study showed mice devoid of the subunit, GluA1, were impaired in working memory suggesting that a greater proportion of AMPA receptors expressed at the surface in the KO mice than the WT mice may contribute to working memory retention (Okun et al., 2010). In contrast, a study examining the role of TLR3 in itch demonstrated that activation of the receptor induced AP firing and inward currents in DRG neurons further eliciting scratching in WT mice however these results were not seen in KO mice. Furthermore, WT mice also increased spontaneous ESPCs showing a further role of TLR3 activation in excitatory synaptic transmission. This would suggest the opposite effect seen in the previous study that WT mice, but not KO mice, would have a greater proportion of AMPARs at the surface (Liu *et al.,* 2012). It is also possible that inflammatory mediators released alter neuronal function. TLR3

activation leads to interferon and inflammatory cytokine release from astrocytes and microglia which may alter the communication between neurons and glia resulting in the disturbance of neuronal excitability (Vezzani *et al*., 2012). Further studies to determine cytokine release in our cultures during TLR3 activation could be carried out by doing a cytokine array or an ELISA.

Furthermore, we have demonstrated the effect of TLR3 activation on neuronal excitability however the data does not suggest whether the effect is directly neuronal or whether it in an indirect effect from another cell through release of gliotransmitters, cytokines or another modulator. One study showed receptors on glial cells can respond to neurotransmitters producing a neuromodulatory response through gliotransmitter release (Araque, 2008). Going forward, this would be an interesting study to undertake and could be done to address this. Electrophysiological studies on action potential firing could be carried out on neuronal cultures or on co-cultures of neurons and astrocytes with the addition of the astrocyte blocker, fluoroacetate to inhibit astrocytic function (Gan et al., 2011). Astrocytes play a major role in the function of neurons including structural support, metabolic support and neurotransmission, it would be reasonable to speculate that the effect on neurons if indirect, would be from astrocytes (Hassel *et al*., 2002).

In summary, the data in this chapter shows that TLR3 is expressed on cell types in primary rat hippocampal cultures and TLR3 activation functionally impairs neuronal activity through reducing spontaneous AP firing.

4. DETERMINING THE MECHANISMS UNDERLYING TLR3-MEDIATED INHIBITION OF SYNAPTIC ACTIVITY IN PRIMARY HIPPOCAMPAL CULTURES

4.1. INTRODUCTION

TLR3 activation has been shown to impair memory retention, adult neurogenesis, neural plasticity, hippocampal dependent learning and promote neuronal survival in the hippocampus as well as behavioural changes in response to viral infections disrupting brain function (Cameron *et al.*, 2007; Lathia *et al.*, 2008; Okun *et al.*, 2011; Patel and Hackam, 2014). Furthermore, it is well established that TLR3 activation exerts its effects through the TRIF-dependent pathway resulting in the release of type 1 IFNs and inflammatory cytokines that can disrupt brain function (Famakin et al., 2011; Ransohoff and Brown, 2012; Verstak *et al.*, 2013). On the other hand, our knowledge of the function of TLR3 in the CNS is very limited despite the fact that it is widely expressed in the CNS (Carty and Bowie, 2011). The involvement of TLR3 in the pathology of neurodegenerative diseases and virally-induced behavioural changes affecting the brain is also under studied as is the consequence of TLR3 activation at the cellular level within the CNS.

The previous chapter demonstrated that TLR3 activation reduced spontaneous AP firing however the mechanisms underlying this effect

are unknown. I have proven my hypothesis that TLR3 activation modulates hippocampal function through alterations in synaptic activity. Therefore the specific aim of the experiments described in this chapter was to investigate the mechanism(s) underlying the effects of TLR3 activation on synaptic activity and to determine if this was mediated through the TRIF-dependent pathway. The experiments were conducted using immunocytochemistry and whole cell patch clamp electrophysiology in voltage clamp mode (see sections 2.2.3 and 2.2.4).

4.2. RESULTS

4.2.1. Short-term TLR3 activation significantly reduces Na^+ current, but not K^+ current in primary hippocampal cultures.

Having demonstrated that both short-term and long-term TLR3 activation suppresses synaptic activity, I went on to investigate possible mechanisms underlying these effects. It has been well established that Na^+ is critical for AP firing in nerve cells (Hodgkin and Huxley, 1952; Yu and Catterall, 2003), which I confirmed using the Na^+ channel blocker, TTX (see chapter 3). Therefore I carried out experiments to determine if modulation of Na^+ channel function was a mechanism by which TLR3 activation inhibited spontaneous AP firing. A significant reduction in Na^+ channel function (869 ± 80pA, n=7, $P < 0.05$) was observed following the application of poly I:C (200µg/ml) in comparison to the naïve cells (1162pA ± 160.2, n=13). In contrast, poly I:C (1µg/ml) had no effect on the peak Na^+ current (1339 ± 256.7pA, n=6, Fig 4.1). No effect on the K^+ current in neurons treated with poly I:C short-term was evident in comparison to naïve neurons (Fig 4.2).

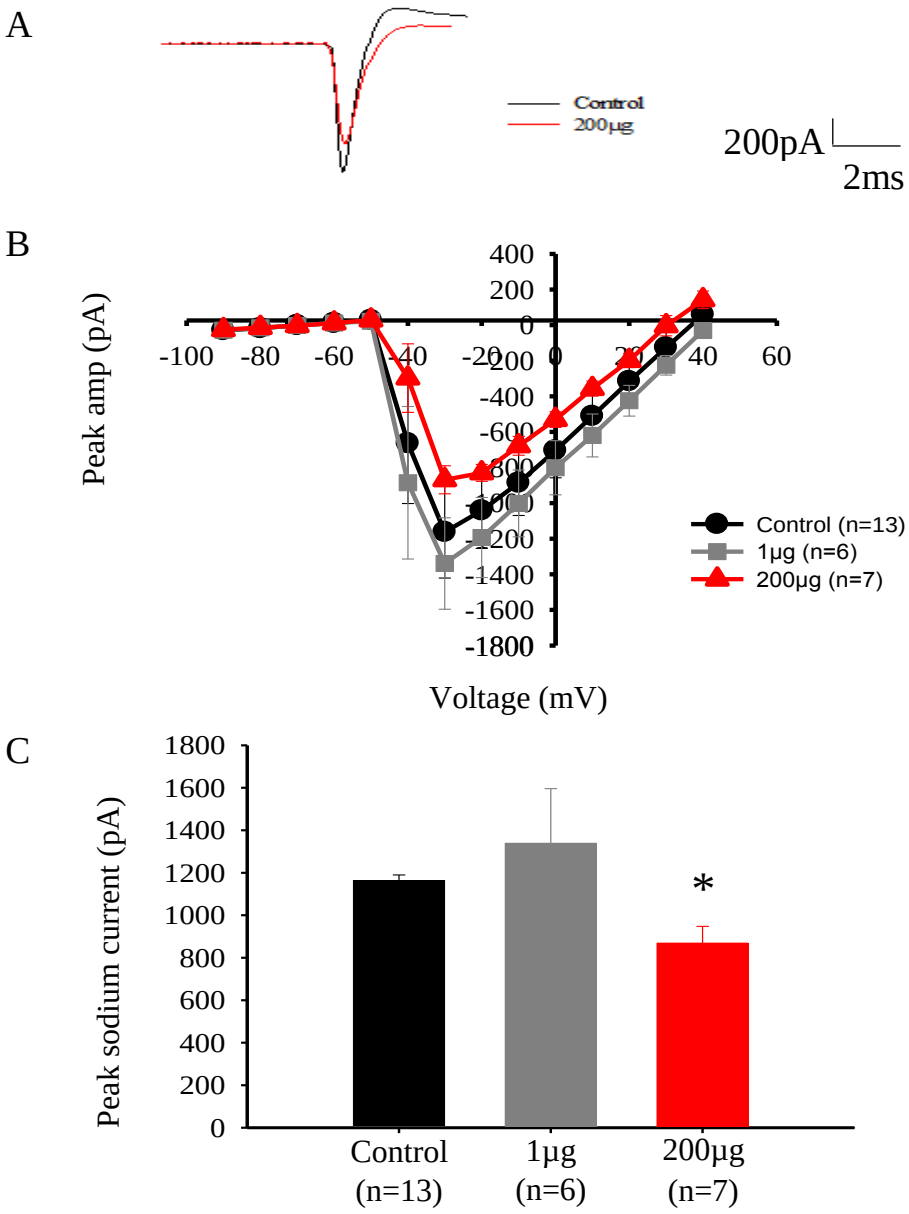


Figure 4.1: Short-term TLR3 activation significantly reduces peak Na^+ current.

A) Representative traces displaying poly I:C (200µg/ml) inhibition of Na^+ current. B) I-V curve revealing effects of poly I:C on Na^+ currents. C) Bar chart summarising the effect of poly I:C on peak Na^+ current, *P< 0.05 using a multiple measure ANOVA with Dunnett's post hoc test and a one way ANOVA with Dunnett's post hoc test for B) and C) respectively. Data are mean ± S.E.M, taken from at least 3 different cultures.

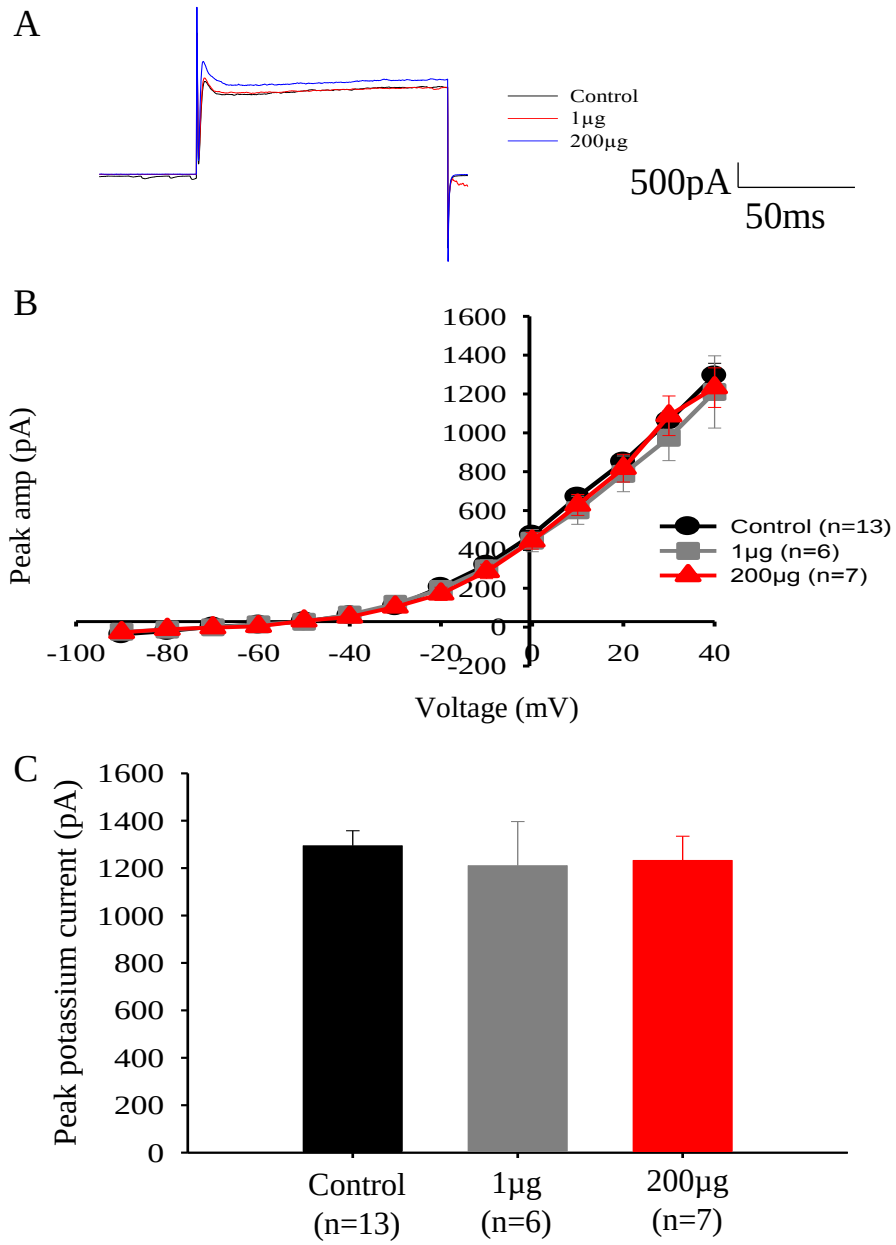


Figure 4.2: Short-term TLR3 activation has no effect on peak K^+ current.

A) Representative traces displaying no effect of poly I:C on peak K^+ currents. B) I-V curve revealing effects of poly I:C on K^+ current. C) Bar chart summarising the effect of poly I:C on peak K^+ current using a multiple measure ANOVA with Dunnett's post hoc test and a one way ANOVA with Dunnett's post hoc test for B) and C) respectively. Data are mean ± S.E.M, taken from at least 3 different cultures.

4.2.2. Long-term TLR3 activation significantly reduces Na^+ current and K^+ current

Short-term TLR3 activation was shown to have an effect on Na^+, but not K^+ channel function, therefore I next examined the consequence of long-term TLR3 activation on Na^+ and K^+ channel function. A significant reduction in the peak Na^+ current (175 ± 70 pA, n=5, P<0.001) resulting from the application of long-term poly I:C (25µg/ml, 1h) was seen in comparison to the maximum peak in naïve neurons (1003 ± 68 pA, n=21) which was reversible (977 ± 72.4 pA, n=8) upon washout (3h). However, no difference was seen in the Na^+ current resulting from poly I:C (1µg/ml) application in comparison to naïve neurons (Fig 4.3). In addition, a +10mV shift in the peak Na^+ channel activation was also evident in treated cultures (naïve peak -30mV vs treated peak -20mV). Moreover, chronic poly I:C (1µg/ml, 1h) and recovery (25µg/ml, 3h) had no effect on the K^+ current in neurons. However a significant reduction in peak K^+ was seen in neurons treated with poly I:C (25µg/ml, 565.2 ± 23.4pA, n=5, P < 0.01) in comparison to naïve neurons (902 ± 223pA, n=14, Fig 4.4).

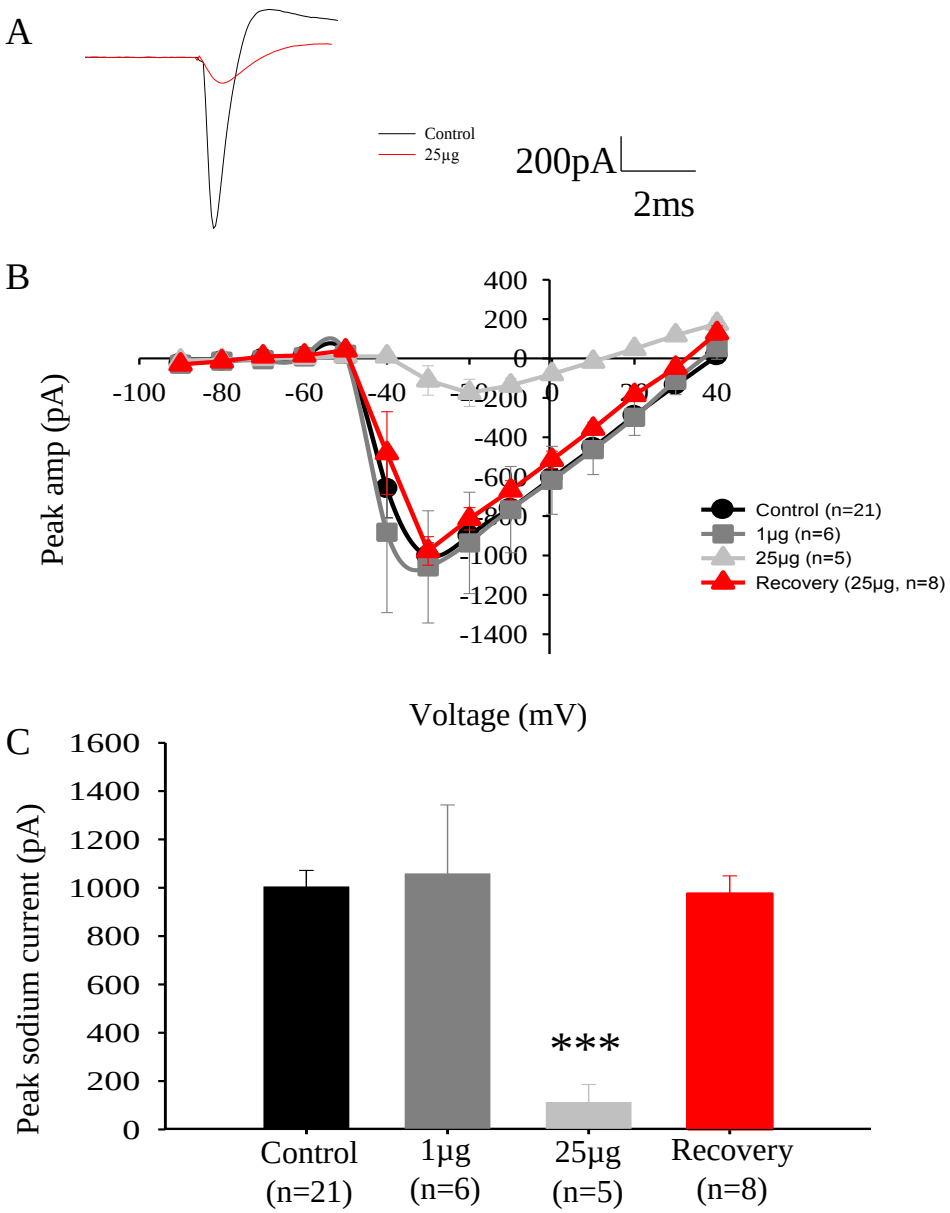


Figure 4.3: Long-term TLR3 activation significantly reduces peak Na^+ current.

A) Representative traces displaying poly I:C (25µg/ml) inhibition of Na^+ current. B) I-V curve revealing effects of poly I:C on Na^+ currents. C) Bar chart summarising the effect of poly I:C on peak Na^+ current, ***P<0.001 using a multiple measure ANOVA with Dunnett's post hoc test and a one way ANOVA with Dunnett's post hoc test for B) and C) respectively. Data are mean ± S.E.M, taken from at least 3 different cultures.

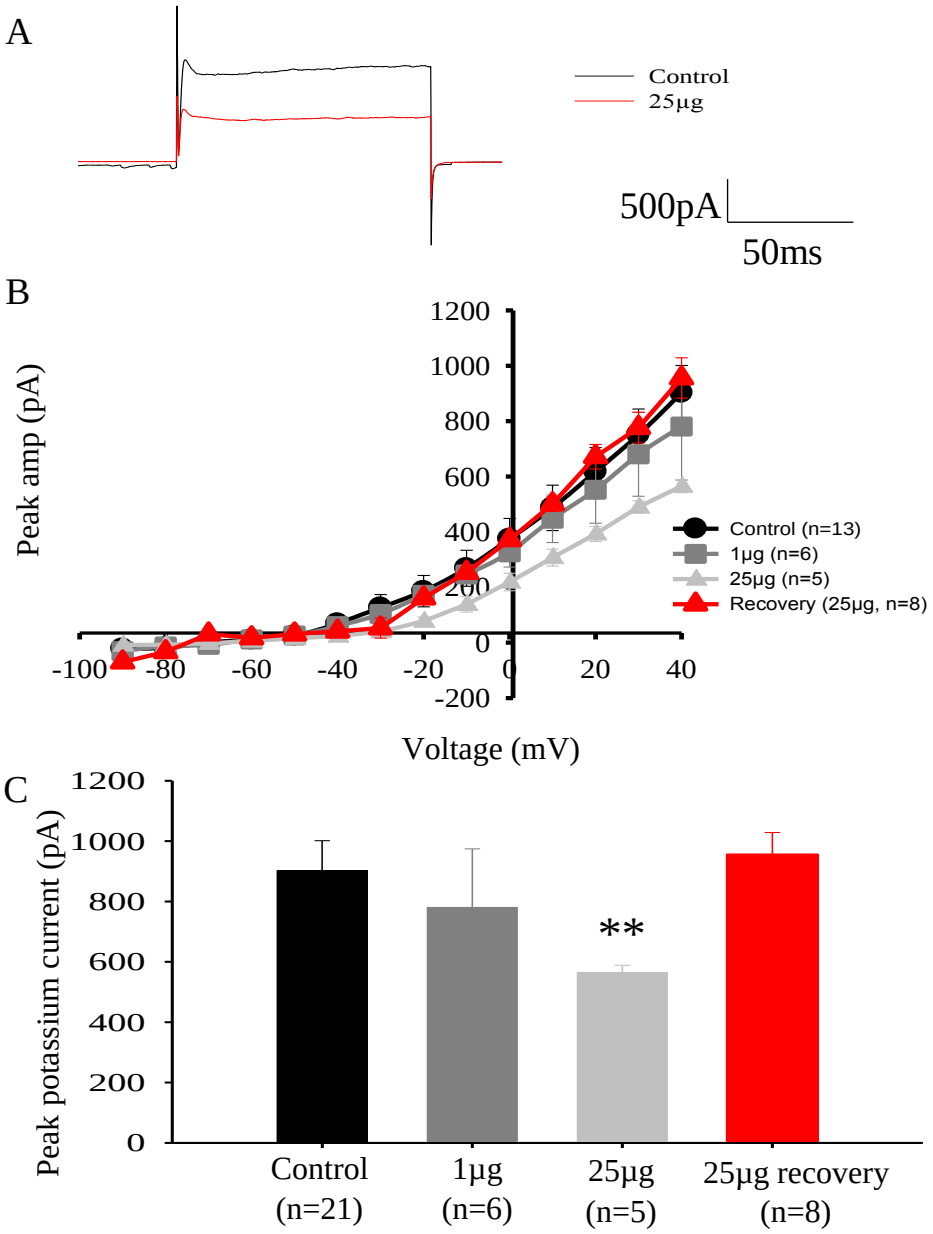


Figure 4.4: Long-term TLR3 activation significantly reduces peak K^+ current.

A) Representative traces displaying poly I:C (25µg/ml) inhibition of K^+ current. B) I-V curve revealing effects of poly I:C on K^+ currents. C) Bar chart summarising the effect of poly I:C on peak K^+ current, **P< 0.01 using a multiple measure ANOVA with Dunnett's post hoc test and a one way ANOVA with Dunnett's post hoc test for B) and C) respectively. Data are mean ± S.E.M, taken from at least 3 different cultures.

4.2.3. Short term TLR3 activation has no effect on the frequency and amplitude of mEPSCs

Having shown that TLR3 activation has a significant effect on sodium function which suggests a possible mechanism of action underlying the effects of TLR3, I also investigated whether TLR3 activation modulates miniature excitatory postsynaptic currents (mEPSCs), which occur in the presence of the Na^+ channel blocker, TTX. Short-term treatment of poly I:C (200µg/ml) had no effect on the frequency of mEPSCs (0.3 ± 0.1 Hz, n=6) in comparison to naïve cultures (0.4 ± 0.1 Hz, n=6, Fig 4.5C). Furthermore, the treatment of short-term poly I:C had no effect on the amplitude of mEPSCs (10.1 ± 1 pA, n=6) in comparison to naïve cultures (11 ± 0.3 pA, n=6, Fig 4.5D).

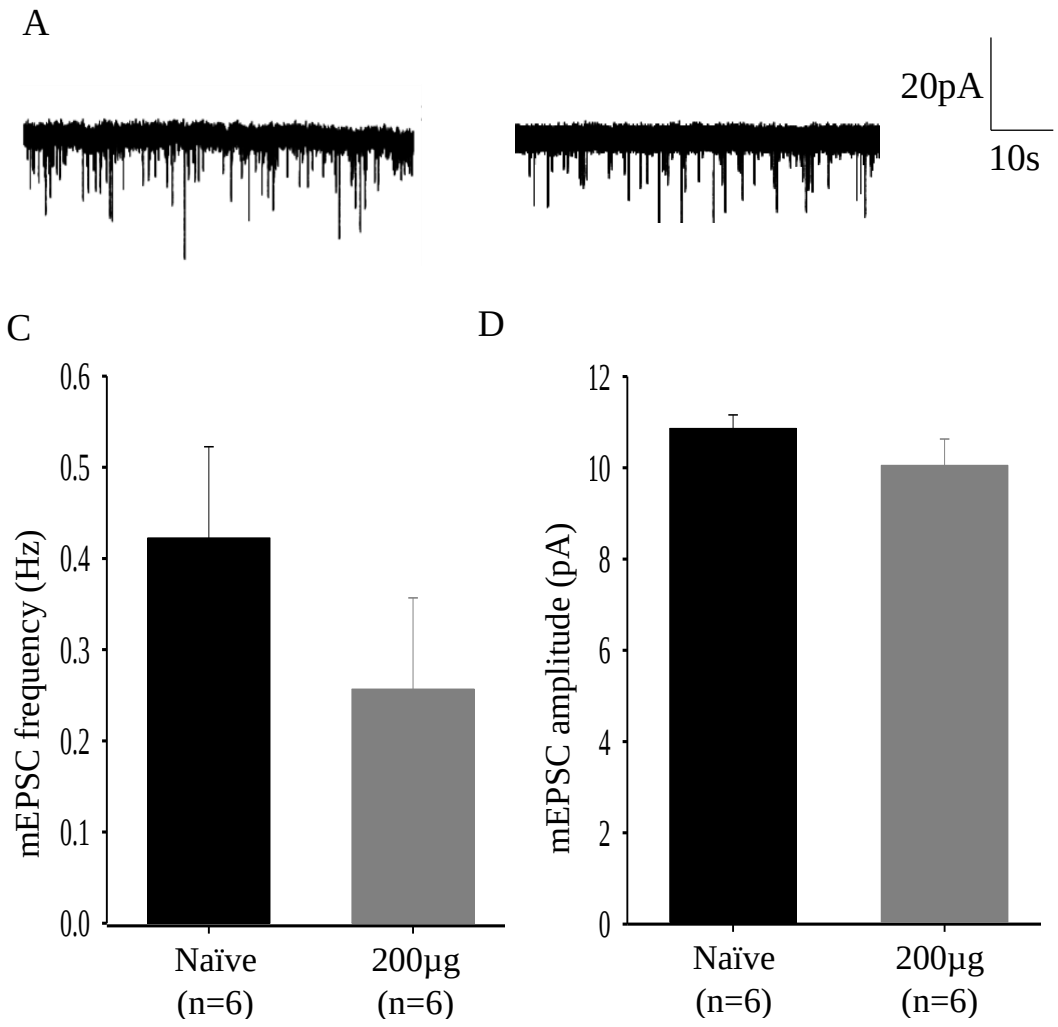


Figure 4.5: Short-term TLR3 activation has no effect on the frequency and amplitude of mEPSCs.

Representative traces displaying mEPSCs in the A) absence and B) presence of poly I:C (200µg/ml). C + D) Bar chart revealing no effects of poly I:C on mEPSC frequency and amplitude respectively using a paired t-test. Data are mean ± S.E.M, taken from at least 3 different cultures.

4.2.4. Long-term TLR3 activation significantly reduces the frequency and amplitude of mEPSCS.

Having demonstrated that no effect was seen on the frequency and amplitude of mEPSCs following short-term TLR3 activation, experiments were carried out to determine the effects of long-term TLR3 activation on the frequency and amplitude of mEPSCs. Chronic treatment of poly I:C (25µg/ml, 1h) significantly reduced the frequency of mEPSCs (0.001 ± 0.0005 Hz, n=7, $P<0.05$) in comparison to naïve cultures (0.4 ± 0.02 Hz, n=9, Fig 4.6C). Furthermore, long-term poly I:C (25µg/ml, 1h) significantly reduced the amplitude of mEPSCs (2.4 ± 1.2 pA, n=7, $P<0.05$) when compared to naïve cultures (8.4 ± 2.0 pA, n=9, Fig 4.6D).

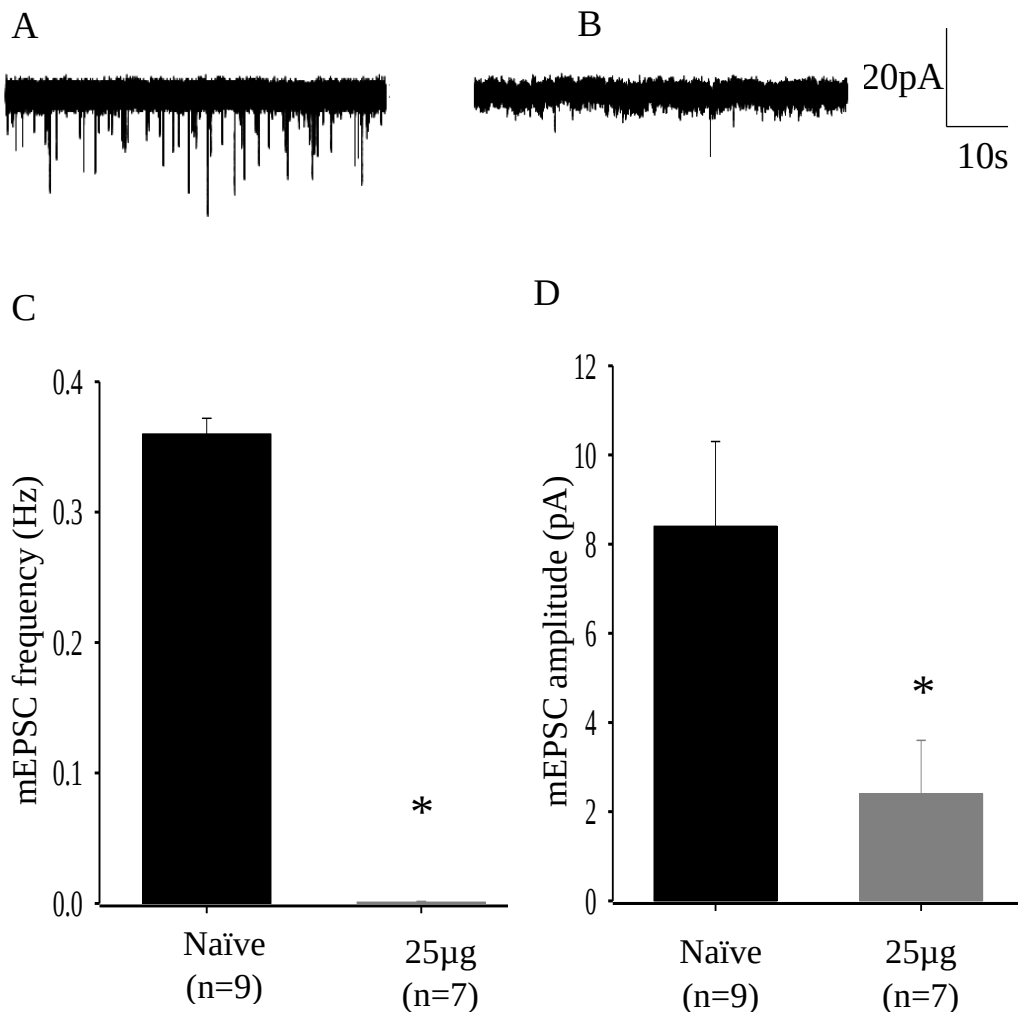


Figure 4.6: Long-term TLR3 activation significantly reduces frequency and amplitude of mEPSCs.

Representative traces displaying mEPSCs in the A) absence and B) presence of poly I:C (25µg/ml). C + D) Bar chart revealing no effects of poly I:C on mEPSC frequency and amplitude respectively, *P < 0.05 using an unpaired t-test. Data are mean ± S.E.M, taken from at least 3 different cultures.

4.2.5. Long-term TLR3 activation significantly reduces surface expression of AMPA receptors

Given that long-term TLR3 activation significantly reduced the frequency and amplitude of mEPSCs from hippocampal neurons, I next investigated the possibility that a reduction in AMPA receptor surface expression accounted for this. There was a significant reduction in AMPA receptor surface expression on neurites after exposure to poly I:C (25µg/ml, 1h), with expression following poly I:C being 58 ± 2% of control (n=97, $P < 0.001$) when compared to AMPA receptor surface expression on neurites in vehicle-treated neurons (Fig 4.7).

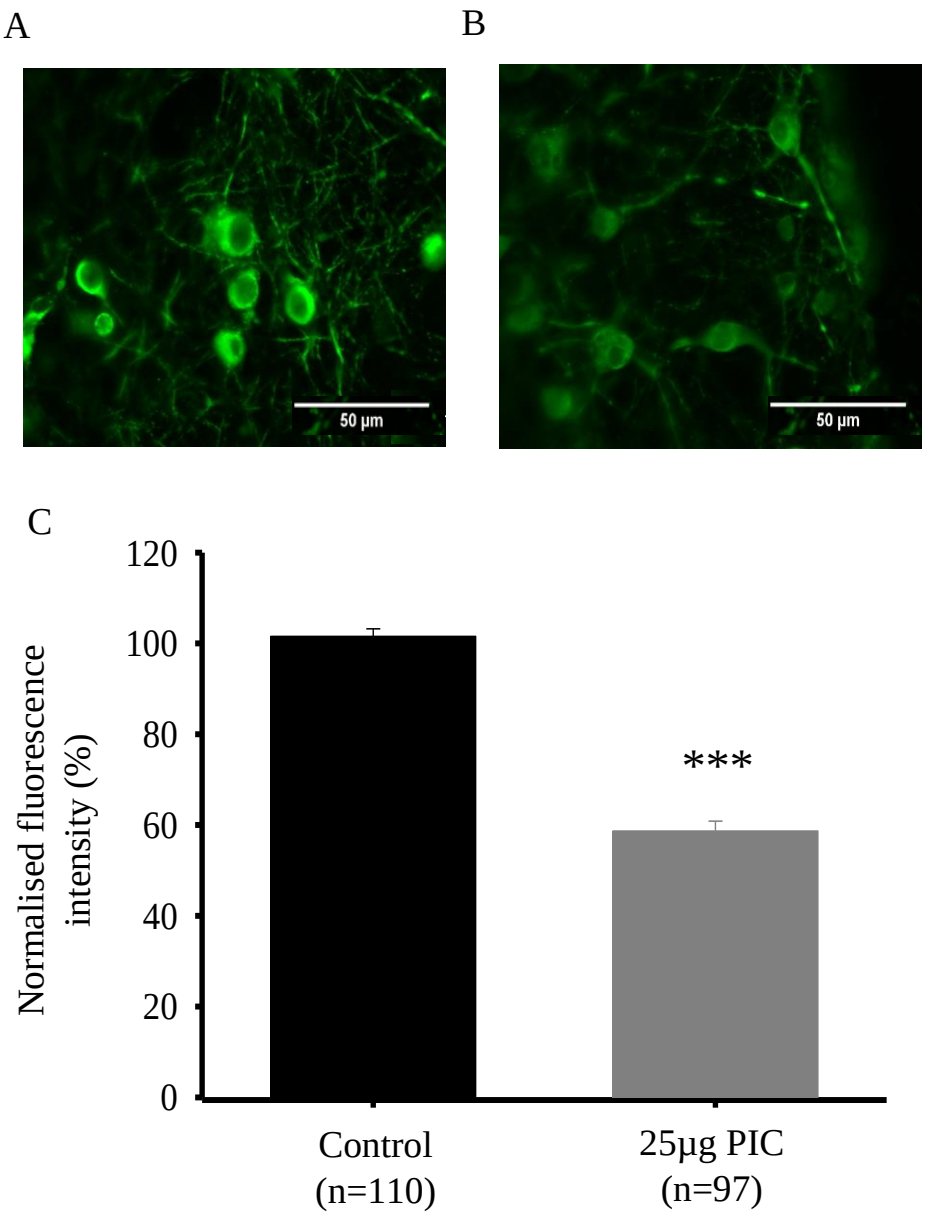


Figure 4.7: Long-term TLR3 activation significantly reduces AMPA receptor surface expression.

Representative images of AMPAR surface expression in A) vehicle-treated and B) poly I:C (25µg/ml,1h) treated cultures. C) Bar chart revealing effects of poly I:C on AMPAR surface expression, ***P < 0.001 using an unpaired t-test. Data are mean ± S.E.M, taken from at least 3 different cultures.

4.2.6. TRIF pathway activation is required for TLR3 inhibition of hippocampal synaptic activity

Demonstrating the effects of TLR3 activation on spontaneous AP firing and the cellular mechanisms underlying these effects led me to carrying out experiments to determine whether the classic MyD88-independent TLR3-TRIF pathway underlies these observations. GFP transfection alone reduced Na^+ currents (602.67 ± 208pA, n=6) when compared to controls (1314 ± 122pA, n=11). However, in agreement with the effect of poly I:C (25µg/ml, 1h) on Na^+ currents (see section 4.2.2), GFP transfected cells treated with poly I:C (25µg/ml, 1h) resulted in a significant reduction in peak Na^+ current (69 ± 68pA, n=6, $P < 0.001$) when compared to their vehicle-treated GFP transfected neurons (602.67 ± 208pA, n=6, Fig 4.8). In contrast, no significant effect was seen in peak K^+ current following poly I:C (25µg/ml, 1h) application (1190 ± 195pA, n=6) when compared to the control (805 ± 183pA, n=6, Fig 4.9). In contrast, neurons co-transfected with a mutant version of TLR3 (A795P), which switches the classical adaptor of TLR3 activation from TRIF to MyD88 (GFP co-transfected to identify transfected neurons) after application of PIC (25µg/ml) showed no effect on peak Na^+ current (1085.8 ± 272.4pA, n=7) in comparison to vehicle-treated controls (1123 ± 243.4pA, n=6, Fig 4.10). In addition, after application of PIC (25µg/ml), no effect on peak Na^+ current was seen (2740 ± 946.4pA, n=7) in comparison to vehicle-treated controls (2133.3 ± 780pA, n=7, Fig 4.11). Studies involving pharmacological intervention to further confirm the TLR3-TRIF pathway involvement were attempted. However, experiments using the TRIF signalling inhibitory peptide (Pepinh-TRIF), which interferes with the interaction between TLR3 and

TRIF, could not be carried out due to the deleterious effects on cell viability.

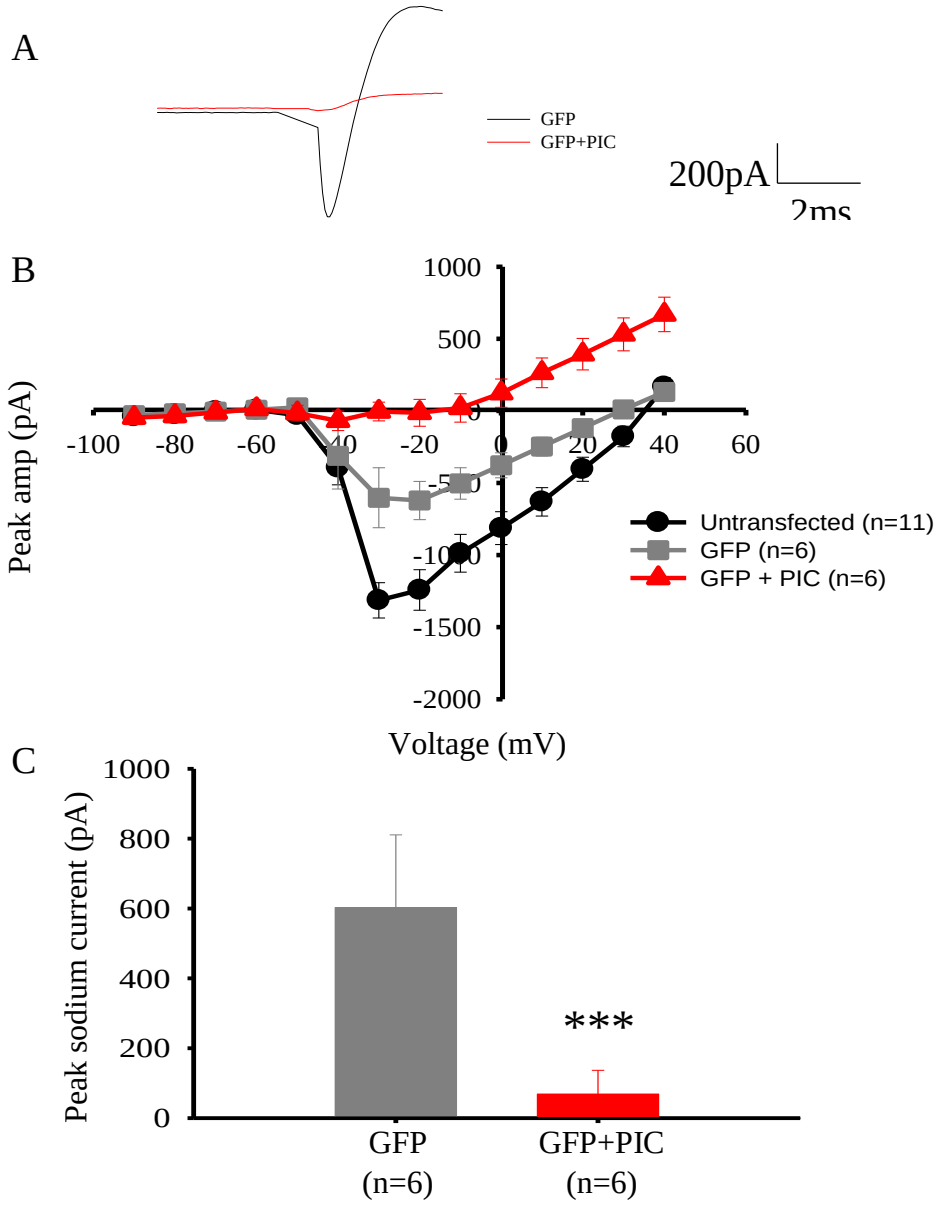


Figure 4.8: Long-term TLR3 activation significantly reduces peak Na^+ current in GFP transfected neurons.

A) Representative traces displaying poly I:C (25µg/ml) inhibition of Na^+ current. B) I-V curve revealing effects of poly I:C on Na^+ currents. C) Bar chart summarising the effect of poly I:C on peak Na^+ current, ***P<0.001 using a multiple measure ANOVA with Dunnett's post hoc test and a one way ANOVA with Dunnett's post hoc test for B) and C) respectively. Data are mean ± S.E.M, taken from at least 3 different cultures.

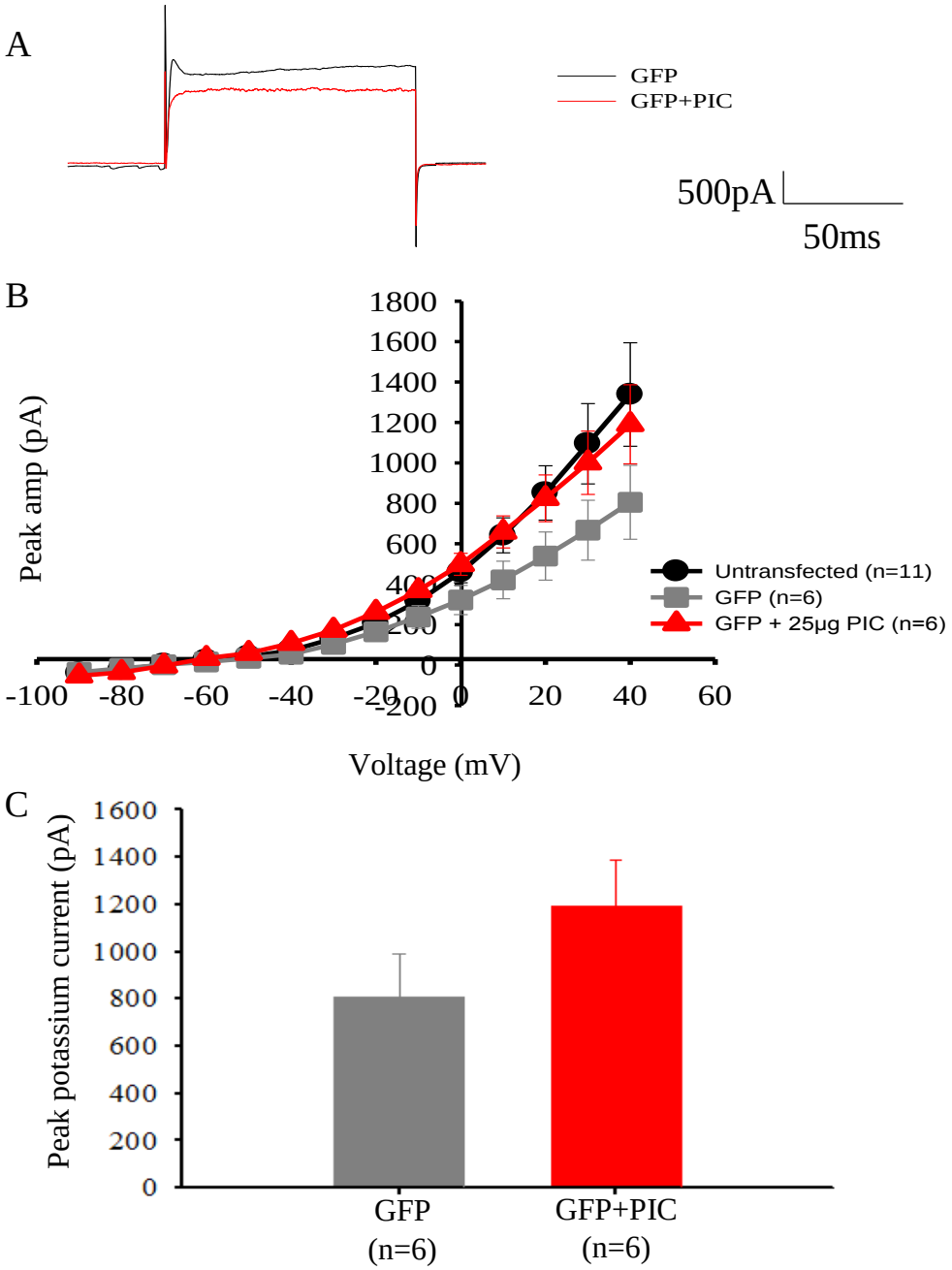


Figure 4.9: Long-term TLR3 activation has no effect on peak K^+ current in GFP transfected neurons.

A) Representative traces displaying no effect on K^+ current. B) I-V curve revealing effects of poly I:C on K^+ currents. C) Bar chart summarising the effect of poly I:C on peak K^+ current using a multiple measure ANOVA with Dunnett's post hoc test and a one way ANOVA with Dunnett's post hoc test for B) and C) respectively. Data are mean ± S.E.M, taken from at least 3 different cultures.

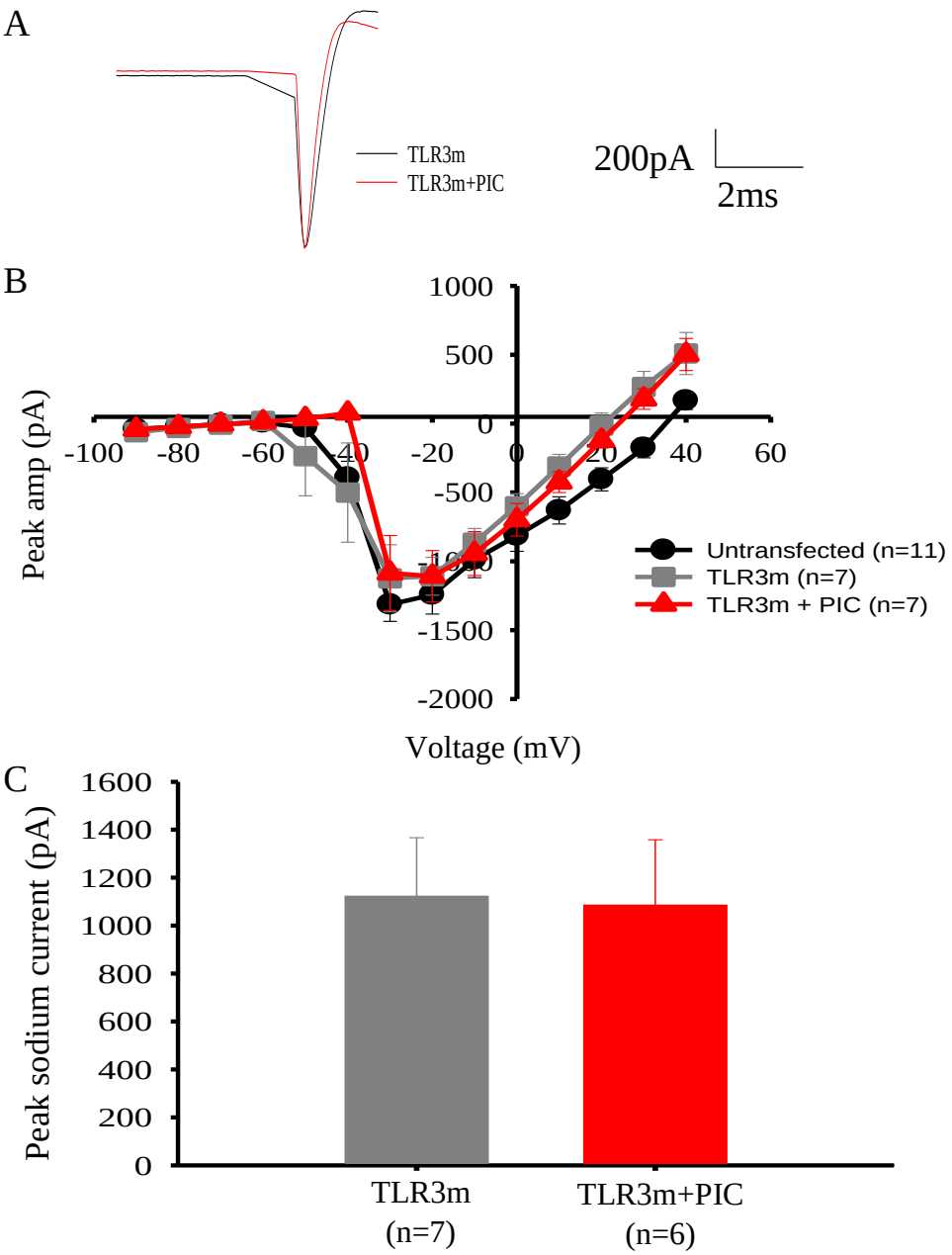


Figure 4.10: Long-term TLR3 activation has no effect on peak Na^+ in TLR3m transfected neurons.

A) Representative traces displaying no effect on Na^+ current. B) I-V curve revealing effects of poly I:C on Na^+ currents. C) Bar chart summarising the effect of poly I:C on peak Na^+ current using a multiple measure ANOVA with Dunnett's post hoc test and a one way ANOVA with Dunnett's post hoc test for B) and C) respectively. Data are mean ± S.E.M, taken from at least 3 different cultures.

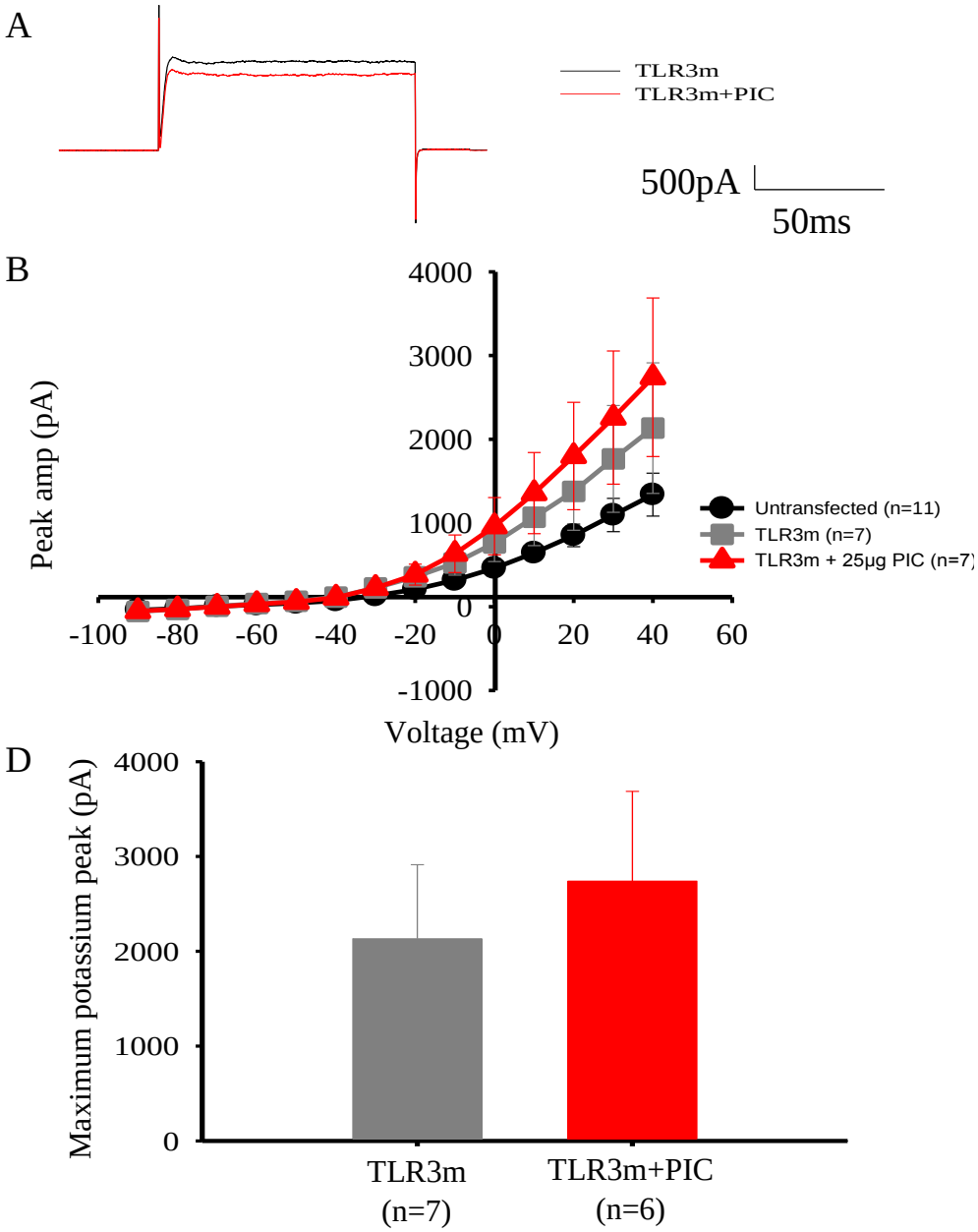


Figure 4.11: Long-term TLR3 activation has no effect on peak K^+ in TLR3m transfected neurons.

A) Representative traces displaying no effect on K^+ current. B) I-V curve revealing effects of poly I:C on K^+ currents. C) Bar chart summarising the effect of poly I:C on peak K^+ current using a multiple measure ANOVA with Dunnett's post hoc test and a one way ANOVA with Dunnett's post hoc test for B) and C) respectively. Data are mean ± S.E.M, taken from at least 3 different cultures.

4.3. DISCUSSION

In this chapter, I demonstrated for the first time the mechanisms underlying the effects of TLR3 activation on spontaneous AP firing. As shown in the previous chapter, TLR3 activation resulted in a decrease in spontaneous AP firing and it is well established that sodium channels are essential for AP firing in nerve cells. However, to the best of our knowledge, no previous studies have shown the effects of TLR3 activation or the mechanisms underlying the effects within the CNS in relation to synaptic activity in neurons. Therefore, I carried out experimental work to determine if modulation of sodium and/or potassium channels was a possible mechanism for the reduction in AP firing seen in primary hippocampal cultures. TLR3 activation following exposure to poly I:C (5 min) had a significant effect on sodium channel function however a significant decrease in both sodium and potassium currents was demonstrated in long-term activation of TLR3 (25µg/ml). In naïve cultures, the maximum influx of sodium into the neuron occurred at -30mV. This suggests that this state of depolarisation is the voltage at which maximum sodium influx occurs before becoming inactive (Hille, 1978). This is reflected in chronic poly I:C treated cultures (25µg/ml) however there is a significant decrease in the amount of sodium influx and there is also a shift in the I-V curve where the maximum peak of sodium influx occurs (-20mV). In addition poly I:C (1µg/ml) does not affect the sodium channel function as the data is identical to naïve neurons. The reduction in Na^+ channel function will have an effect on neuronal excitability and subsequently the firing of APs (Pristerà *et al.,* 2012). To the best of our knowledge, this is a novel finding and suggests that sodium channels aren't functioning to full

capacity. However, it has been shown that an enzyme that cleaves a subunit of Na^+ channels leads to a loss of function resulting in an impairment in neuronal excitability (Kim *et al.*, 2007). In agreement, another study showed that a mutation in Na^+ channels decreases the excitability of the channels which results in neurons expressing this mutant channel requiring a higher threshold to initiate AP firing therefore reducing neuronal excitability (Barela *et al.*, 2006). As previously mentioned, spontaneous AP firing in neurons is sodium channel dependent therefore the effects seen by TLR3 activation may be caused by sodium channels being inhibited. An alternative explanation may be that a reduced density of the channels prevent depolarisation as a previous study revealed a link between neurotrophins and sodium channel density. Indeed this study showed that dorsal root ganglion neurons exposed to neurotrophin 3 resulted in the reduction of sodium channel expression (Wilson-Gerwing et al., 2008). As previously mentioned, neurotrophins are growth factors essential for development of the CNS, however data has recently shown that TLRs can act as neurotrophic receptors therefore it is possible that neurotrophins may be involved in how TLR3 reduces synaptic activity (McIlroy *et al.*, 2013). Therefore it would be intriguing to determine whether the connection between neurotrophins and TLR3 holds true in hippocampal cultures. Application of chronic poly I:C (25µg/ml) significantly decreased the potassium current in neurons compared to naïve neurons. This would generally lead to an increase in excitability as the neuron is incapable of repolarising back to resting potentials however this cannot account for the decrease in AP firing. Sodium channels open causing further depolarisation of the cell membrane resulting a feedback loop where potassium channels then open allowing an efflux of potassium ions

enabling the membrane potential to return to resting state (Eijkelkamp *et al.*, 2012; Luan *et al.*, 2014).

To determine if TLR3 activation affected neurotransmission at the synaptic level, I next investigated the effect on the quantal vesicle release by conducting mEPSC experiments. I did this by adding TTX, which blocks sodium channels and therefore leads to the cease of spontaneous AP firing allowing mEPSCs to be investigated (Wall and Usowicz, 1998). I determined if mEPSC frequency and amplitude was altered during TLR3 activation thus identifying that TLR3 activation results in changes at the synaptic level. Short-term activation of TLR3 had no effect on frequency or amplitude of mESPCs showing that TLR3 activation does not cause changes at the synaptic level. Therefore this finding suggests that the inhibition of sodium channel function is the mechanism underlying reduced spontaneous AP firing in response to short term TLR3 activation. In contrast, long-term activation of TLR3 significantly reduced both the frequency and amplitude of mEPSCs. As previously mentioned, a change in the frequency suggests that the mechanism through which TLR3 is acting may be presynaptic (Han and Stevens, 2009). However alteration in amplitude suggests postsynaptic effects, which include NMDA receptor involvement or alterations in AMPA receptor surface expression (Turrigiano, 2012). In agreement, one study showed that TLR3 KO mice had an increased level of AMPA receptor surface expression at the dendritic spine membrane of the postsynaptic neuron in comparison to WT mice. This suggests that TLR3 may be regulating the expression of AMPA on the surface through constitutive internalisation and this may be an additional mechanism to explain how TLR3 acts (Okun et al., 2012). Therefore it could be argued

that the study shows both presynaptic and postsynaptic effects and it is reasonable to speculate that a postsynaptic effect may explain why frequency is also reduced. The explanation for this is that if there is a change of AMPARs at the post-synaptic terminal resulting in reduced expression, this in turn will give rise to negative feedback loop which enables synaptic strength to be altered through a change in the probability of neurotransmitters being released in a homeostatic plasticity mechanism termed synaptic scaling.

To determine whether AMPA receptor internalisation accounts for the changes seen in long term activation of TLR3, GluA1 expression was measured as per previous studies (Moult *et al.,* 2010). AMPA receptor surface expression was significantly reduced implying that TLR3 activation modulates AMPA receptor expression on hippocampal neurites. Thus, it is reasonable to speculate that this reduction may account for the reduction in both frequency and amplitude of mEPSCs and as previously mentioned, this finding is consistent with previous studies (Okun *et al.,* 2010). However, this finding contradicts the DRG study which showed synaptic transmission was impaired in TLR3 KO's (Liu *et al.,* 2012). This data supports our hypothesis that TLR3 activation modulates hippocampal function and therefore the results carried out in this chapter provide further insight into the mechanisms underlying the reduction in synaptic activity in response to TLR3 activation. Further studies using super ecliptic pHluorin-GluA1 (SEP-GluA1) to monitor AMPA receptor surface expression or measuring this through mRNA or protein expression and ligand binding may be useful (Gocel and Larson, 2013).

Studies examining the signalling pathways involved in TLR3 activation using transfection as a method were carried out on neurons. I wanted to determine if the effects of TLR3 activation were mediated via the TRIF pathway, the classical signalling pathway used by TLR3. These were carried out by using neurons transfected with GFP for controls and transfecting neurons with a mutant version of TLR3 (A795P) to study the effect of TLR3 activation. The mutant version enhances the activation of NF-κB whilst being incapable of mediating IRF3 responses which are dependent on TRIF (Verstak *et al*., 2013). Neurons transfected with GFP alone acted as controls for this experiment and results showed that TLR3 activation significantly reduced sodium channel function. Further experiments would be required to determine the effect this result has on synaptic activity and spontaneous AP firing. In contrast, the mutant version of TLR3 preserved sodium channel function in response to TLR3 activation suggesting that the underlying effects of TLR3 activation are dependent on TRIF pathway activation as the mutant version of TLR3 has switched adaptor specificity from TRIF to MyD88 (Verstak *et al*., 2013). To the best of our knowledge, this is a novel finding. Furthermore, studies using the inhibitory peptide (Pepinh-TRIF), which blocks the TRIF pathway by interfering with the interaction between TLR3 and TRIF shown in other studies (Zhang *et al.,* 2011; Hosmane *et al.,* 2012; Gambara *et al.,* 2015), was carried out to pharmacologically confirm the underlying effects of TLR3 activation requires TRIF pathway activation. However due to deleterious effects on the cells, studies were unable to be carried out.

In summary, in this chapter, I demonstrated for the first time that TLR3 activation reduces sodium and potassium channel function. Furthermore,

short-term TLR3 activation has no effect on mEPSC frequency and amplitude whereas long-term TLR3 activation reduced both frequency and amplitude of mEPSCs. My findings indicate that the reduction of frequency and amplitude of mEPSCs is due to a reduction of AMPA receptor surface expression. In addition, the underlying effects of TLR3 activation are dependent on TRIF pathway activation.

5. GENERAL DISCUSSIONS

The main purpose of this thesis was to investigate whether TLR3 activation in the hippocampus modulates AP firing, synaptic transmission, the mechanisms underlying the effects and determine the subcellular localisation of TLR3 on cell types within primary hippocampal cultures. The central hypothesis of this thesis was that TLR3 activation modulates hippocampal function through changes in synaptic activity. It was hoped that by examining AP firing, synaptic transmission and the mechanisms underlying these effects, such *in vitro* investigations would provide valuable insights about the role of TLR3 activation in the CNS and provide key evidence underlying behavioural changes in the CNS mediated by viral infections. Thus, in this final chapter, I will firstly summarise the major findings in this study. Next, I will discuss the significance of this study in relation to established knowledge of TLR3 in the CNS. Lastly, based on the data I obtained, I will suggest some future studies.

5.1. Major findings

The major findings in this thesis are listed below:

Firstly, from immunocytochemistry experiments on primary hippocampal cultures.

1. Neurons, astrocytes and oligodendrocytes expressed TLR3.
2. The presence of TLR3 on neurons was found to only be intracellular.

3. Long-term TLR3 activation significantly reduced AMPA receptor surface expression at neurites.

Secondly, from immunocytochemistry experiments on human neuronal cultures.

1. Human neurons expressed TLR3.
2. The presence of TLR3 on human neurons appeared to be found both intracellularly and extracellularly on the cell surface.
3. Other cells within the culture expressing TLR3 are proposed to be neural progenitor cells.

Lastly, from functional experiments in primary hippocampal cultures.

1. Spontaneous AP firing in neurons was reduced in the application of short-term TLR3 activation.
2. Reduction found in spontaneous AP firing in short-term TLR3 activation recovered during the washout stage.
3. Spontaneous AP firing in neurons was reduced in long-term TLR3 activation application.
4. Recovery of spontaneous AP firing after long-term TLR3 activation (1µg/ml PIC) was increased however was not significant.
5. Recovery of spontaneous AP firing 18 hours after long-term TLR3 activation (1µg/ml PIC) was significantly increased.
6. Recovery of spontaneous AP firing an hour after long-term TLR3 activation (25µg/ml PIC) was significantly increased.
7. Long-term poly A:U application (25µg/ml) significantly reduced spontaneous AP firing.

8. Long-term LPS application (25µg/ml) had no effect on spontaneous AP firing.
9. Peak sodium current but not peak potassium current was reduced after short-term TLR3 activation.
10. Peak sodium current and peak potassium current were reduced after long-term TLR3 activation.
11. No effect on mEPSC frequency or amplitude was seen after short-term TLR3 activation.
12. Frequency and amplitude of mEPSCs was significantly reduced after long-term TLR3 activation.
13. Peak sodium current was significantly decreased in GFP transfected cells after long-term TLR3 activation.
14. No effect was seen on peak sodium and peak potassium current in TLR3m transfected cells after long-term TLR3 activation.

5.2. TLR3 localisation differs between neurons in hippocampal cultures and human neurons

The immune system and CNS has been shown to communicate through cross-talk of CNS cells with roles in homeostasis and neurogenesis (Okun *et al*., 2010; Peferoen *et al.*, 2014). As studies have shown TLR3 to be expressed on all cell types and localised on murine and human neurons (Trudler, 2010), initial experiments were performed to confirm this in addition to examining the subcellular localisation of TLR3 in neurons, astrocytes, oligodendrocytes and microglia within primary hippocampal cultures and also in human neurons. Here I showed that in

agreement with previous studies, TLR3 is expressed intracellularly on both cultured neurons and human neurons however expression of TLR3 on human neurons was also on the plasma membrane. This finding suggests that there may be differences in TLR profile and localisation between murine and human neurons which would be useful in future studies when carrying out experiments to gain more knowledge on processes and mechanisms that occur under different pathophysiological conditions in the human brain. In addition iPSCs present the possibility of their usefulness in the research field and for regenerative medicines. Deriving the cells is a slow and inefficient process and there has been some concerns over the safety of using these cells for clinical applications, for example, the potential of tumours forming however developing methods and strategies have progressed in providing solutions to the challenges faced by using iPSCs (Boland *et al*., 2009; Li *et al.*, 2011). Most importantly, studying them in disease contexts better relates to the consequences of diseases in an individual and refines the use of animal work. It would be worth carrying out functional experiments on human neurons by using electrophysiology to look at neuronal activity after TLR3 activation, the mechanisms underlying the effects and the pathway used.

5.3. TLR3-mediated inhibition of spontaneous action potential firing

A novel finding from my thesis is that in primary hippocampal cultures, TLR3 activation led to a reduction in spontaneous AP firing with recovery upon washout. I firstly demonstrated that application of a

sodium channel blocker and a cocktail of glutamate receptor antagonists completely prevented the generation of APs. Taken together, this data suggests the occurrence of APs in cultured neurons are sodium dependent and synaptically driven. To the best of my knowledge, neuroexcitability in response to TLR3 activation has not been extensively studied. However, one study did show that systemic administration of poly I:C into mice modulated hippocampal excitability by increasing the likelihood of epileptic activity (Costello *et al.,* 2013). In agreement, another study demonstrated that TLR3 played a role in the induction of seizures (Benninger *et al.,* 2013) whereas a study looking at TLR3 in itch also provided evidence that TLR3 activation induced inward currents and action potential firing (Liu *et al.,* 2012) which is in disagreement with my results. However, these studies were carried out in models of epilepsy, DRG neurons and slices therefore suggesting under certain conditions and dependent on specific neuronal type, TLR3 may respond in a different way.

During synaptic transmission, studies have observed cross-talk between neurons and glia (Auld and Robitaille, 2003; Vezzani *et al*., 2012). We have demonstrated the effect of TLR3 activation on neuronal excitability however the data does not suggest whether the effect is directly neuronal or whether it in an indirect effect from another cell through release of gliotransmitters, cytokines or another modulator. One study showed receptors on glial cells can respond to neurotransmitters producing a neuromodulatory response through gliotransmitter release (Araque, 2008). To address this issue, electrophysiological studies on action potential firing should be carried out neuronal cultures or on co-cultures of neurons and astrocytes with the addition of the astrocyte blocker,

fluoroacetate (Hassel *et al*., 2002). In addition, the release of interferon beta has been shown to modulate neuroexcitability in neocortical pyramidal neurons *in vitro* (Hadjilambreva *et al.*, 2005) therefore release of interferons or cytokines may play a role in hippocampal neuroexcitability. It could provide useful to determine if cytokines and/or interferons were released into the media of primary hippocampal cultures and whether or not they affect AP firing. This could be studied through cytokine arrays and ELISAs to determine if cytokines and/or interferons are released in response to TLR3 activation and electrophysiological studies using the released cytokines/interferons, if found using respective techniques, to determine the effect they have on AP firing,

The inhibition of spontaneous AP firing in response to TLR3 was not reliant on changes in membrane potential as this study showed that no effect on membrane potential in TLR3 activated neurons was seen when compared to controls. Therefore I studied alternative mechanisms to explain the reduction in AP firing.

5.4. Multiple mechanisms underlie TLR3-mediated effects on spontaneous action potential firing

Next, I investigated alternative mechanisms to explain the reduction of spontaneous AP firing seen in response to TLR3 activation. First, as AP generation in this study was shown to be sodium dependent, I carried out experiments to determine if sodium channel function was a possible mechanism underlying the AP firing inhibition. I demonstrated that

TLR3 activation inhibited sodium channel function which, to the best of my knowledge, is a novel finding. An alternative TLR, TLR4, has been shown to increase neuronal excitability and sodium current densities in sensory neurons when activated (Due *et al.*, 2012) which is the opposite of the TLR3-mediated effects. If TLR4, responsive to bacteria, increases neuronal excitability and sodium current densities then it could be entirely plausible that TLR3, responsive to viruses, decreases neuronal excitability and inhibits sodium channel function. Studies have demonstrated that in the fruit fly, TLR6 and TLR7 are involved as neurotrophic receptors (McIlroy *et al*., 2013) and involvement of neurotrophin-3 has been shown to reduce sodium channel expression (Wilson-Gerwing *et al.*, 2014). In addition, it has been shown that some TLRs once activated including TLR3 and TLR4, stimulate neurotrophin expression from astrocytes therefore there is a possibility of neurotrophin involvement (Li *et al*., 2012). It may provide useful to carry out an assay to determine if any neurotrophins are expressed in response to TLR3 activation and examine their effect on neuronal functions. Although my data supports the explanation that a reduction in sodium channel function leads to the inhibition of AP firing, alternative mechanisms could still exist because this mechanism demonstrated may not be the sole contributing factor.

It was previously shown that TLR3-deficient mice had elevated levels of AMPARs in the CA1 region of the hippocampus providing the possibility of a reduction in amplitude of mEPSCs which would suggest a postsynaptic effect (Okun *et al.*, 2010). However, another study disagreed by showing poly I:C increased the frequency, but not the amplitude of sEPSCs. Therefore I looked at the involvement of synaptic

transmission providing an explanation to AP firing inhibition. I demonstrated that short-term TLR3 activation had no effect on synaptic transmission suggesting that sodium channel function may be the sole contributing factor however long-term TLR3 activation significantly reduced the frequency and amplitude of mEPSCs suggesting a postsynaptic effect. I then showed that a decrease in AMPAR surface expression resulted from TLR3 activation which is in agreement with Okun's work. However this does not rule out other postsynaptic mechanisms including changes in NMDAR expression or activity. Further experiments to determine the involvement of NMDARs should be carried out.

Having demonstrated mechanisms underlying TLR3-mediated spontaneous AP firing inhibition, I then determined if the classical TRIF pathway activated in response to TLR3 activation underlies the functional changes seen in neurons. Another novel finding in my thesis demonstrated an involvement of the TRIF pathway underlying the effects of TLR3 activation. I showed, through the use of a mutant version of TLR3 which switches from using the adaptor molecule TRIF, to MyD88, that the effects of TLR3 activation on sodium channel function was blocked.

5.5. Involvement of TLR3-dependent hippocampal modulation on viral-mediated behavioural changes and the possible consequences of these effects

As previously mentioned, TLR3 has been shown to inhibit neurogenesis, axonal growth, affects learning and memory, increase the risk of psychiatric disorders in offspring due to maternal infection and play a role in infectious disorders such as CNS viral infections and non-infectious disorders including stroke and some neurodegenerative diseases (Cameron *et al*., 2007; Field *et al.,* 2010; Okun *et al*., 2010; Ciu *et al.,* 2013). However there is not a substantial amount of evidence to help us understand the involvement of TLR3-dependent hippocampal modulation of viral-mediated behavioural changes and the consequences of these effects.

One study showed that during maternal infection, an imbalance between inhibitory and excitatory neurotransmission occurs due to TLR3 activation which significantly affects network function and may contribute to behavioural changes, cognitive deficits and psychiatric disorders later on in adulthood (Ebert and Greenberg, 2013). In agreement, it was demonstrated that maternal influenza infection could increase the risk of schizophrenia in the offspring later in adulthood through the increase in serotonin system and a decrease in the glutamate system which induce schizophrenia like psychosis (Moreno *et al.*, 2011). These data support our finding of a reduction in excitatory neurotransmission however it is still unknown whether this reduction in activity we see in our lab has a long-term effect later on in life.

Furthermore, enhanced TLR activation including TLR3, TLR4, TLR7 and TLR8/9 in response to pregnancy-associated malaria led to an increase in a variety of cytokines including TNF-α, IL-6 and IL-10. This increase in cytokines was shown to be associated with an increased likelihood of the offspring being infected with the parasite which causes malaria, P.falciparum, at birth (Gbédandé *et al.,* 2013). This suggests that the immune response is already being conditioned which may compromise the ability of the immune system in the offspring to fight against further infections in the future. Moreover, the release of cytokines so early in life may have a negative effect of the developing brain and could be a vulnerability factor contributing to the cognitive defects, behavioural changes and increased risk of psychiatric defects later on in life. In agreement, a study demonstrated that inducing TLR3-mediated immunity during gestation resulted in cortical neurogenesis inhibition and behavioural disturbances (Miranda *et al.,* 2010).

Furthermore, these behavioural changes have been linked to depression and shown to disrupt brain function which can also be passed to an unborn child. (Blomström *et al.,* 2015; Reisinger *et al.,* 2015). An association between depression and the identification of depression-related interferon-inducible genes (DRIIs) was discovered in patients receiving interferon alpha therapy. Furthermore, TLR3 activation and murine IFN resulted in the expression of these genes and inflammatory cytokines in hippocampal neurons and it has been suggested that these along with neural plasticity inhibition may play a role in IFN-associated depression (Hoyo-Becerra *et al*., 2013). These behavioural changes are proposed to be caused by the release of inflammatory mediators,

cytokines and interferons mainly, and can vary dramatically between individuals (Dantzer *et al.*, 2010; Maes *et al.*, 2012).

It is still not fully understood whether the results seen are a form of neuroprotection and the relationship between inflammation and neurodegenerative diseases. There has been multiple studies that show TLR3 activation to have both neurotoxic and neuroprotective properties, depending on the context and environment the receptor is activated (Patel and Hackam, 2014).

A recent hypothesis has emerged stating that inflammation resulting from viral infections has the ability to prime neurons and immune cells in the CNS, contributing to neuronal populations being susceptible to degeneration in response to insults. In turn, this leads to chronic neuronal dysfunction which initiates neurodegenerative diseases (Deleidi and Isacson, 2012). In agreement, an increase in proinflammatory cytokine release has been demonstrated in early onset of neurodegenerative diseases with the immune system activation being shown to be involved in the degeneration of neurons in Huntingtons disease (Björkqvist *et al.*, 2008; Song *et al.*, 2009). There are some studies proving that TLR3 in response to viral infections induces neurodegeneration however knowledge is still limited on whether TLR3 plays a role in the pathogenesis of neurodegenerative diseases (Field *et al.*, 2010; Qin and Crews, 2012). Furthermore one study has suggested that lifestyle can affect the likelihood of neurodegeneration later on in life. It was demonstrated that excessive amounts of alcohol increases neuroinflammation and degeneration in response to systemic TLR3

activation through cytokine release and microglial neurodegenerative markers (Qin and Crews, 2012).

Taken together, the data from my study suggests that TLR3 activation modulates hippocampal function through neuronal excitability and provides mechanisms of action including sodium and potassium channel function, neurotransmitter release, AMPA internalisation and the requirement of TRIF pathway activation. These findings clearly provide a significant insight into the function of TLR3 in the CNS and how these functional changes may correlate to viral-mediated behavioural changes.

5.6. Future studies

The results detailed in this thesis have opened up the possibilities of investigation of TLR3 in CNS development and function. Based on the investigations discussed, there are still some interesting questions that need to be answered and several further experiments are obviously needed. The following are some of the unresolved questions that may be worth investigating:

1. Is TLR3 activated reduction in synaptic activity truly TLR3-dependent? This could be investigated by carrying out electrophysiological experiments using an agonist for each TLR receptor.
2. Do all neurons (glutamatergic and GABAergic) within cultures express TLR3? Immunocytochemical studies could be carried out

to determine this and provide further insight into effects seen on TLR3 activation.

3. TLR3 activation showed recovery short-term and long-term however full recovery was not seen in long-term TLR3 activated cells. Are cells damaged or do they require longer to recover? Electrophysiological experiments allowing cells to recover for longer than 24 hours should be carried out to confirm. If full recovery is not present after this, cell viability assays could be carried out to determine if cells are damaged or dying.
4. Does inflammatory cytokine release affect neuronal function? Cytokine array or an ELISA could be done to confirm.
5. Is the effect on neuronal excitability a direct effect on neurons or indirect? To address this issue, electrophysiological studies on action potential firing should be carried out neuronal cultures or on co-cultures of neurons and astrocytes with the addition of the astrocyte blocker, fluoroacetate.
6. Are neurotrophins involved in the functional changes? This could be determined through electrophysiological experiments in response to neurotrophin or an assay could be used to determine if any neurotrophins are produced in response to TLR3 activation.
7. TLR3 was shown to be expressed on human neurons. Do we see the same functional changes in human neurons in response to TLR3 activation as was seen in cultures? This could be investigated through electrophysiological studies.

PUBLICATIONS

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Ritchie, L., Paul, A and Bushell T.J. (2014). The activation of TLR3 results in the inhibition of synaptic activity in primary hippocampal cultures, SIPBS Research Annual Symposium, University of Strathclyde.

Ritchie, L., Paul, A and Bushell T.J. (2014). The activation of TLR3 results in the inhibition of synaptic activity in primary hippocampal cultures, Postgraduate Research Day, University of Strathclyde.

Ritchie, L., Paul, A and Bushell T.J. (2015). TLR3 modulation of hippocampal synaptic activity, SIPBS Research Annual Symposium, University of Strathclyde.

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