Age-Related Changes in the Phenotype of Microglia

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Declaration

All of the experiments and analysis detailed in this thesis has been completed exclusively by myself and has not previously been submitted for any other degree or qualification.

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Publications

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Abstract

Microglia play a central role in immune surveillance and modulation of neuroinflammation as well as playing a role in neurodevelopment. Microglia involve in the development of pathological pain in adults but not early in life. However little detail is known about the changing phenotype of microglia during development. We examined age-related changes in microglia following activation with pathogen- and damage- associated molecular patterns (PAMPs/DAMPs). Microglial cultures were prepared from neonatal postnatal day (P1) and adult (P40) rat brains and spinal cords. Immunocytochemistry, qRT-PCR and functional assays were used to identify age-related differences. Adult microglia display a pro-inflammatory immune profile characterized by significantly increased IL-1β mRNA levels in response to PAMPs and DAMPs. In contrast, IL-1β mRNA in neonatal microglia showed a slight increase after stimulation with DAMPs. Anti-inflammatory gene expression was significantly increased in neonatal microglia relative to adult microglia. Compared to adult microglia, neonatal cells had increased phagocytic activity when unstimulated and following activation with LPS and ATP. Moreover, the nuclear receptor Nurr1 may play a major role in reducing pro-inflammatory signalling and promoting the anti-inflammatory phenotype in neonatal microglia. Nurr1 isoforms are differentially expressed in neonatal and adult microglia, with the Nurr1a isoform being significantly elevated in neonatal cells. Using lentiviral vector-mediated expression of Nurr1 isoforms, we also show that over-expression of TINUR, a splice variant of Nurr1, in neonatal and adult microglia attenuates inflammation by trans-repression the IL-1β expression and trans-activation the IL-10 gene expression following ATP exposure. Together, these data provide evidence for age-related difference in microglial function during postnatal development. In addition, these findings demonstrate insight into the

mechanisms by which Nurr1 might act, and suggest potential therapeutic targets for the treatment of neuro-inflammatory diseases.

1 Chapter one: General Introduction

1.1 Pain and nociception

The International Association for the Study of Pain defines pain as ‘an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in term of such damage’. Pain includes a group of complex experiences that involve the transduction of noxious stimuli through to cognitive and emotional processing by the central nervous system (CNS) (Julius and Basbaum, 2001). The perception of and responses to pain are dependent on many factors such as memories, personality, sensory inputs, motor activity and genetic make-up (Casey, 1996). Nociception, a significant component of the overall pain experience, is the detection of noxious stimuli translated into nerve impulses (action potentials) that are sent to the spinal cord and then to brain centers which interpret them and initiate responses, both motor and emotional (Godfrey, 2005).

1.2 Anatomy of pain pathways

1.2.1 The primary afferent nociceptors

Somatosensory information sensed by peripheral sensory receptors is sent to the CNS through primary afferent fibres. The primary afferent neurons can be activated by irritant chemicals and noxious heat (Burgess and Perl, 1967). These sensory fibres originate from cell bodies in trigeminal and dorsal root ganglia (DRG), and are classified based on structure, cell body diameter and action potential conduction velocity into three main groups Aβ, Aδ and C fibres (Millan, 1999).

Myelinated Aβ fibres are large in diameter (>10μm) with high conduction velocity (30-100m/s) (Djouhri et al., 1998). Aβ fibres are responsive to innocuous stimuli and do not contribute to the detection of noxious inputs (Lawson, 2002). Aδ fibres have a

medium diameter (2-6µm) and are finely myelinated with intermediate conduction velocities (5-25m/s). These fibres are activated by high threshold noxious stimuli (Clancy and McVicar, 1998). C-fibres are smaller in diameter (0.4-1.2µm), unmyelinated and have slower conduction velocity (1-2m/s) (Monafo, 1995). Peripheral C-fibre terminals act as polymodal nociceptors which are sensitive to a wide range of stimuli including chemicals, heat and intense mechanical perturbation (Julius and Basbaum, 2001).

Polymodal nociceptors contain a multitude of specialized receptors and ion channels that can transduce the noxious stimuli into signals in the pain sensing neurons. Classification of primary afferent nociceptors can also be based on their neuroanatomical and molecular characterization, particularly for C-fibres (Snider and McMahon, 1998). For example, two populations of C-fibres can be discerned, peptidergic and non-peptidergic sub-types. The peptidergic fibres release neuropeptides such as calcitonin-gene related peptide (CGRP); these fibres also express tyrosine kinase receptor type I (TrkA), which is a receptor for nerve growth factor (NGF). The second C-fibre population is non-peptidergic, expressing the c-Ret neurotrophin receptor that is sensitive to glial-derived neurotrophic factor (GDNF). Moreover, these fibres express the adenosine tri-phosphate (ATP)-gated ion channel P2X3 (Molliver and Snider, 1997, Dong et al., 2001). All primary afferent fibres release many neurotransmitters to the dorsal horn including, glutamate, nitric oxide (NO), ATP, cannabinoids, opioids and phospholipid metabolites such as prostaglandins (Kontinen and Meert, 2002, Meyer et al., 2006).

The sensation of pain is of two forms; fast, sharp pain which is associated with Aδ fibres and slow, dull pain that is mediated through C fibres (McHugh and McHugh, 2000, Julius and Basbaum, 2001). The activation of the nociceptors is modulated by many chemical messengers such as histamine, substance P and acetylcholine that are released when tissue damage occurs. These mediators influence nerve activity and the intensity of pain sensation. Repeated stimulation of C fibres by noxious stimulus can lead to increased excitability of spinal neurons; this phenomenon has been termed central sensitization (Basbaum et al., 2009), which is a key element of chronic pain.

1.2.2 Dorsal horn (DH) of the spinal cord

Primary afferent fibres make synaptic connections with second-order neurons in the DH, which is the first integration site of somatosensory information within the CNS. The grey matter of the spinal cord contains the nerve cell bodies of spinal neurons. It is subdivided into ten laminae (Rexed, 1952, Rexed, 1954). Aδ fibres project mainly to lamina I and some of them terminate in laminae V and X whereas C fibres terminate mostly in laminae II and weakly innervate laminae I, V and X (Cross, 1994). However, laminae III to IV receive projections mainly from Aβ fibres which function largely as mechanoreceptors (Sugiura et al., 1986).

DH neurons can also be categorized according to their response to nociceptive input into three types; nociceptive specific, non-nociceptive and wide dynamic range (WDR) neurons. Nociceptive specific are found mainly in laminae I and II, and they are high threshold neurons, activated by noxious stimuli and receive signal from Aδ and C-fibres. Non-nociceptive neurons are located in laminae II, III and VI, and they are activated by innocuous inputs. However, WDR neurons can be found in laminae

IV, V and X and they respond to a variety of stimuli such as heat, noxious chemicals and pressure. WDR neurons respond to activity that is mediated by all three kinds of primary afferent fibres (Cross, 1994, Bromm and Lorenz, 1998).

1.2.3 Ascending pain pathways

The Spinothalamic (STT) and spinoreticular tracts (SRT) are two major ascending pathways that carry pain information to the thalamus and brain stem, respectively (Willis Jr, 1985). The STT originates mainly from neurons in laminae I and V-VII, but also from laminae X (Millan, 1999, Craig et al., 2001). Three forms of afferents of the STT tract have been defined based on the projection of STT fibres; two of them are monosynaptic pathways including the ventral spinothalamic tract directly projecting to nuclei of the lateral complex of the thalamus, which has a role in the sensory components of pain. Other monosynaptic spinothalamic pathway projects to medial central nucleus of thalamus and is related to the components of the painful experience. Finally, a multi-synaptic paleospinothalamic pathway directs to nuclei of the posterior medial of the thalamus and participates in the motivational affective aspects of pain (Zhang et al., 1990, Dostrovsky and Craig, 2006).

The cells of the spinoreticular tracts originate mainly in laminae V, VII and VIII (Willis and Westlund, 1997, Millan, 1999). Spinoreticular tract projections in the brain stem are projected towards the precerebellar nucleus in the lateral reticular formation and are participated in motor control. Other projections target the medial pontobulbar reticular formation, which has a role in the mechanisms of nociception (Millan, 1999). Many spinoreticular afferents respond to noxious stimuli and neuro-vegetative responses to pain. Furthermore, this tract is an important pathway for brain stem

responses including activation of endogenous analgesia systems and descending control of spinal processing (Zhang et al., 1990, Foote et al., 1991, Millan, 1999).

1.2.4 Descending pain pathways

Networks of descending pathways project from cerebral structures to exert both inhibitory and facilitatory influences on the DH of the spinal cord and, thereby, control the transmission of nociceptive information to the brain (Millan, 2002). The midbrain regions, rostroventromedial medulla (RVM) and periaqueductal grey (PAG) are crucial for the descending control of pain. The PAG integrates inputs from different areas such as the thalamus, hypothalamus, cortex, spinal cord and amygdala. The PAG sends projections to the RVM that, in turn, project down through the dorsal lateral funiculus terminating in the dorsal horn to reduce pain transmission (Stamford, 1995). The PAG-RVM system has been identified as the crucial site of action of analgesic agents including opioids and cannabinoids (Millan, 2002, Maione et al., 2006). The balance between the descending inhibitory and facilitatory pathways is subject to change following injury and an imbalance has been involved in development of chronic pain (Moffat and Rae, 2011). Furthermore, the link of PAG to serotonergic and non-serotonergic elements of the RVM and noradrenergic neurons in the medulla are important in modulating the descending system (Mason, 1999, Odeh and Antal, 2001).

1.3 Pathophysiology of pain pathways

1.3.1 Peripheral inflammation and pain

Inflammation constitutes the body's physiological response that is triggered by noxious stimuli, such as infection and tissue injury and allows clearance of harmful

agents and repair of injuries to restore homeostasis (Medzhitov, 2008, Maskrey et al., 2011). Inflammation may release or generate various pro-inflammatory mediators such as serotonin, bradykinins, prostaglandins, histamines, cytokines and nitric oxide (NO). These mediators contribute to inflammation signs including, heat (calor), redness (rubor), pain (dolor), swelling (tumor) (Aguzzi et al., 2013).

Inflammation is central to the development of many diseases including cancers, autoimmune, cardiovascular and neurodegenerative diseases (Serhan et al., 2010). Acute inflammation is the early response of the immune cells against pathogens and tissue injury. It is a rapid process, facilitated by eicosanoids and vasoactive amines which increase the flow of plasma and leukocytes into infected tissue (Serhan et al., 2008, Medzhitov, 2008). However, various cytokines and growth factors are released in chronic inflammation resulting in recruitment of immune cells including leukocytes, fibroblast and lymphocytes that lead to persistent tissue damage. Chronic inflammation occurs in neurodegenerative disorders, including Parkinson's disease (PD) and Alzheimer's disease (AD), and it is associated with the homeostatic imbalance of at least one of many physiological systems (Medzhitov, 2008, Aggarwal et al., 2009). Chronic inflammation in the CNS or the neuroinflammation concept is used to describe a set of conditions that are specific to the CNS.

1.3.2 Neuropathic and inflammatory Pain

Neuropathic and inflammatory chronic pain are debilitating conditions in which the pain experience continues upon nerve injury or painful stimulus (Tsuda et al., 2009). Chronic pain models have shown that glial cells play an essential role in persistence of pain and its chronic nature (Sweitzer et al., 2001). Chronic inflammatory pain is

related to inflammatory diseases, including arthritis, where the presence of inflammatory mediators leads to nociceptor sensitization in damaged tissue (Ringkamp et al., 2013). Neuropathic pain is a condition that occurs in response to nerve damage, which is stimulated by infection, cancer, autoimmune disease, diabetes and trauma (Baron, 2006).

The most common feature of neuropathic pain is hyperalgesia, which is an increased sensitivity to pain, that can be separated into primary and secondary hyperalgesia. Increases sensitivity to an afferent nerve stimuli (peripheral sensitization) causes primary hyperalgesia, which leads to increased nociceptive transmission from the periphery and modulates neuronal activity in the dorsal horn. Secondary hyperalgesia is an increase a response in undamaged tissue close to the area of actual tissue damage, which results in increased excitability of CNS neurons (central sensitization) (Millan, 2002, Kuner, 2010). Animal models show that neuropathic pain leads to increased excitability in the dorsal horn neurons as well as a loss of the efficacy of the inhibitory neurotransmitters including gamma-Aminobutyric acid (GABA) and glycine in inhibitory interneurons (Scholz and Woolf, 2002, Prescott et al., 2014). Moreover, peripheral nerve injury (PNI)-induced hyper-excitability might cause multiple functional alterations in microglia besides changes in neurons (Watkins et al., 2001, Suter et al., 2007).

1.3.3 Neuroinflammation

Neuroinflammation is a term used to describe the inflammatory reactions in the CNS in response to pathological conditions or increased neuronal activity (Xanthos and Sandkühler, 2014). Neuroinflammation preserves homeostasis to help the CNS deal

with metabolic stresses and increases the plasticity of neuronal networks (Ransohoff and Brown, 2012). However, neuroinflammation may also become maladaptive and amplify the effects of pain, epilepsy and stress (Milligan and Watkins, 2009, Vezzani et al., 2011). This intricate integration of responses include microglia, astrocytes, endothelial cells, interneurons and infiltrating T-lymphocytes, all of which play a crucial role in neuroinflammation by generating both harmful and useful effects (Sierra et al., 2015, Benraiss et al., 2016). Astrocytes are found exclusively in the CNS and they are the most abundant glial cells offering critical functional support for neurons, including reuptake of excessive glutamate and potassium and production of cytokines and transmitters (Volterra and Meldolesi, 2005, Oberheim et al., 2012). On the other hand, astrocytes may also participate in neuroinflammation through cross-talk with microglia to increase the immune response and induce dopaminergic neuronal death via activation of apoptotic mechanisms (Saijo et al., 2009a). In addition, the infiltrated T-lymphocytes that enter the brain play a crucial role in initiating neuroinflammation in PD models (Reynolds et al., 2007).

Microglia make up the immune system of the CNS, and although they represent 10-20% of the CNS cellular population, they play a big role in cellular-immunology and neuroinflammation, as well as acting as the first line of defence against infection or injury (Streit and Kincaid-Colton, 1995, Nimmerjahn et al., 2005). Microglial responses are mediated via several effector mechanisms, including clearance of the debris or extracellular aggregates, initiation of the repair process by stimulating the astrocytes to produce trophic factors and release many neurotrophins and anti-inflammatory cytokines (Kim et al., 2009, Neumann et al., 2009). Neurodegenerative diseases including, multiple sclerosis, PD and AD are related to chronic

neuroinflammation and increased levels of some cytokines (Chiu et al., 2012). Chronic neuroinflammation includes persistent activation of microglia and successive continued release of inflammatory mediators in addition to increased oxidative stress (Tansey et al., 2007, Frank-Cannon et al., 2009). In general, microglia activation is an important component of neuroinflammation as it contributes to neuronal dysfunction, injury and disease progression. Therefore, without microglia activation and neuroinflammation, recovery from CNS injuries and removal of invading pathogens might be compromised. The subsequent sections will detail the structure and function of specific cell types in more detail.

1.4 Glia and glial cell types

Glial cells have a supporting function to neurons, regulating the ionic environment and removing debris (Silver et al., 2015). It is well established that glial cells are the intimate partner of neurons throughout their lifespan (Castonguay et al., 2001). There is mounting evidence that the neuron-glia intercommunication plays a crucial role in pain, synaptic plasticity and neuronal functions (Vernadakis, 1996, Milligan et al., 2003). In addition, glial cells do express numerous receptors for a variety of neurotransmitters and neuromodulators, plus synthesise and release various mediators (Colburn and DeLeo, 1999, Milligan et al., 2003). The cell types that constitute the glial cell population are astrocytes, microglia and oligodendrocytes which form approximately 70% of the total cells in CNS.

1.5 Microglia

Microglia are known as resident macrophages of the CNS. They can be distinguished from other glial cells by their morphology, origin and gene expression functions

(Ransohoff and Perry, 2009, Ginhoux et al., 2010, Perry et al., 2010). At rest, microglial cells display a characteristically ramified morphology with branching processes actively sensing their microenvironment (Sołtys et al., 2001). Once activated, the cells proliferate and change morphology from a ramified to an amoeboid shape and acquire specific functions associated with synaptic pruning, phagocytosis of apoptotic neurons and release of inflammatory cytokines (Tsuda et al., 2005, Paolicelli et al., 2011, Sierra et al., 2015). To assess microglial activation, there is an increase in expression of markers which are specific to microglia, such as integrin alpha M (ITGAM), major histocompatibility complex II (MHC II) and ionised calcium binding adapter molecule 1 (Iba-1) (Eriksson et al., 1993).

1.5.1 Origin of microglia

The developmental origin of microglia has been a subject of argument for many years since del Rio-Hortega (del Rio-Hortega and Penfield, 1932) first described microglial cells. It is now accepted that microglial cells are derived from myeloid-monocytic cells and their hematopoietic precursors. Cells of myeloid origin or their precursors enter the CNS at early stage of development (embryonic day 8), then migrate and differentiate to colonize the CNS parenchyma (Kaur et al., 2001, Chan et al., 2007).

1.5.2 Microglia morphology and distribution in the CNS

Microglia are distributed throughout the CNS and they are distinct in morphology across CNS regions (Harry and Kraft, 2012). Remarkably, the microglial morphology and density are region specific, for example, microglia are highly abundant in the gray matter compared to the white matter. Moreover, ramified microglia in the gray matter show round somata, branched processes and small amount of perinuclear cytoplasm,

whereas in white matter they display oval somata, few processes and the cells line up their cytoplasmic extensions in parallel to nerve processes (Savchenko et al., 2000, McKay et al., 2007, Olah et al., 2011). The microglial differences throughout the CNS are related to the microenvironment and functional and morphological heterogeneity, including receptor expression patterns, expression of different cytokines and the immune response (Kaur and Ling, 1991, Olah et al., 2011).

1.5.3 Physiological functions of microglia

1.5.3.1 Phagocytosis and synaptic pruning

Phagocytosis is pivotal for the clearance of apoptotic neurons, deleterious metabolic products and cellular debris in addition to maintaining tissue homeostasis, preventing autoimmune response and resolving inflammation (Napoli and Neumann, 2009, Sierra et al., 2015). As microglia form the first line of defense, phagocytosis is a typical feature of activated microglia. Microglia are attracted by endogenous and exogenous chemotactic factors and migrate toward infection or injury in the CNS (Miyake et al., 2015). To sense the signal that is associated with tissue injury, inflammation and other mediators, microglial cells increase the expression of specific receptors, allowing them to move through CNS tissue (Benveniste, 1997, Hanisch, 2002).

Throughout CNS development, degenerating phenomena together with naturally occurring cell death are common. Microglial cells are known to act as phagocytes responsible for clearance of apoptotic neuronal cells during CNS ontogeny (Ferrer et al., 1990). Moreover, synaptic remodelling occurs throughout the life span of an organism and microglial phagocytosis contributes to the removal of synaptic materials (Paolicelli et al., 2011). Several studies assigned microglial cells an important role in

this aspect during CNS development. Microglia express complement receptors, which are pivotal for pruning (Fourgeaud and Boulanger, 2007, Stevens et al., 2007).

Several cytokines including interferon (INF)-γ, tumour necrosis factor (TNF)-α, Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 are able to impact the microglial phagocytic activity. Furthermore, exogenous stimuli such as lipopolysaccharide (LPS) can also control cellular debris uptake (Chan et al., 2003). The deficiency in phagocytic activity of microglia has been related to pathogenesis of age-related diseases (Sokolowski and Mandell, 2011). Increased production of cellular debris and inflammatory factors in the aged CNS may supress the phagocytic activity of microglia (Cunningham et al., 2013). Moreover, microglia phagocytic mechanisms may occur through aberrant signalling of the neuronal chemokine, fractalkine (CX3CL1), or to its receptor (CX3CR1) on microglia (Hughes et al., 2002). Depletion of microglial CX3CR1 receptor in stimulated adult microglia may result in decreased phagocytic activity of microglia (Koscsó et al., 2016).

1.5.3.2 Antigen presentation

Microglial cells are the major antigen-presenting cells (APC) in the CNS parenchyma. MHC class I and II molecules are expressed by microglia at low levels (Höftberger et al., 2004, Stoll et al., 2006). However, upon infectious neuropathology, autoimmune and neurodegenerative diseases, these molecules are up-regulated together with the costimulatory molecules CD11a, CD45, CD58 and CD86, all of which are crucial for appropriate APC function and T cell stimulation (Kreutzberg, 1996).

1.5.3.3 Production of immune-regulatory molecules

1.5.3.3.1 Chemokines

Chemokines are chemotactic cytokines expressed in CNS cells and tissues and play a central role in intercellular communication and cellular migration in normal tissue (Locati and Murphy, 1999, Zhang et al., 2000). In addition, neuro-inflammatory processes, brain tumors, multiple sclerosis and neurodegenerative diseases such as AD, all seem to involve chemokine actions through constitutive and up-regulated chemokine receptors (Nishioku et al., 2010). Once the microglial cells are activated, they express and secrete a wide spectrum of chemokines. Also chemokines can be upregulated in microglia during many circumstances including CNS development such as CXCL1 (Filipovic et al., 2003), multiple sclerosis such as CCL3 (Balashov et al., 1999), ischemia such as CXCL8 (Popivanova et al., 2003), AD such as CCL2 (ISHIZUKA et al., 1997), and bacterial and viral infections such as CCL5, CCL4 (McManus et al., 2000, Strack et al., 2002). Moreover, microglia can affect and communicate with neurons and astrocytes via chemokines (Nau and Brück, 2002).

1.5.3.3.2 Cytokines

Cytokines, are another group of crucial signalling molecules for microglia, including IL-1, IL-2, -3, -4, -6, -10, -12 and TNF-α. These small molecules are usually expressed and secreted by microglia during inflammation. Important endogenous factors, which can act as inflammatory mediators, are able to induce cytokines release in microglia, including lipids, serum proteins, platelet-activating factor (PAF), complement factors, ATP and High mobility group box 1 (HMGB1) (Hanisch, 2002). Exogenous inducers which cause secretion of cytokines in microglia include viral envelope proteins, LPS, bacterial DNA and prions (Heppner et al., 2001).

IL-1 and TNF-α are crucial microglial effector pro-inflammatory/immune-stimulatory cytokines with various influences and many common functions. Following neuroinflammation, IL-1 is rapidly released and is mainly involved in initiation of neuroinflammation by its ability to increase the expression of chemokines and adhesion molecules that are important for the attraction of leukocytes (Lee and Benveniste, 1999, Hanisch, 2002). IL-1 is a common connection in numerous processes resulting in neuronal death, and inhibition of endogenous IL-1 production exerts neuroprotective effects (Rothwell and Luheshi, 2000). Moreover, IL-1 is involved in many processes during CNS development including, cell proliferation and differentiation (Giulian et al., 1988). Additionally, IL-1 mediates synaptic efficiency in neuronal cells (Vitkovic et al., 2000) and regulates neuro-endocrine-immune circuits (Tringali et al., 1998). Microglia express IL-1 receptor (IL-1R1), which suggests that a paracrine feedback loop can establish to amplify the injury signal by allowing the IL-1 released from microglia to stimulate more microglial activation (Basu et al., 2004). Furthermore, IL-1 also triggers astrocytes to produce GM-CSF, which effect the activation of microglia (Lee et al., 1994).

Alternatively, microglia can also release many anti-inflammatory cytokines, including IL-10, -4 and -13, all of these share anti-inflammatorily features, immune-suppressive and neuroprotective actions. The outcomes of these cytokines can be assigned to a down-regulation of microglial production of pro-inflammatory cytokines such as IL-1, TNF-α, chemokines and redox oxygen species (ROS) (Aloisi et al., 1999, Yuan and Neufeld, 2001, Rasley et al., 2006).

1.5.4 The role of microglia in neuropathic and inflammatory pain

Microglia can respond to the subsequent release of mediators from damaged neurons in the DH following peripheral injury. In addition, microglial cells express a variety of receptors for the neurotransmitters that are released from the primary afferent terminals, including glutamate, N-methyl-D-aspartate (NMDA) and AMPA receptors, purinergic receptors (P2X7 and P2X4) and TrkB receptors for brain derived neurotrophic factor (BDNF) (Ransohoff and Perry, 2009, McMahon and Malcangio, 2009). Electrical activation of primary afferent C-fibres in the sciatic nerve causes changes in sensory processing as a result of increased activation and changed distribution of microglia in the DH. Moreover, blockade of peripheral nerve conduction inhibits nerve injury-induced microglial activation (Hathway et al., 2009b, Suter et al., 2009). The number of microglial cells increases following injury in the DH of the spinal cord and shows more amoeboid features with thick retracted processes and hypertrophied soma (Wen et al., 2007). In addition, the expression of genes that are related to immune responses in microglia undergo alterations following injury; for instance, MHC II is increased antigen presentation, synthesis and release of various mediators including TNF and IL-6, chemokines (MCP-1 and CCL2), ROS and NO (Wen et al., 2007, Hathway et al., 2009b).

Purinergic signalling pathway in activated microglia and BDNF release are essential in the increase of neuropathic pain (Burnstock, 2008). ATP is able to activate microglia through purinergic receptors (Honda et al., 2001, Moss et al., 2007). It was reported that the expression of P2X4 receptors was increased in microglia after PNI, and that PNI-induced allodynia was attenuated by pharmacological inhibition of this receptor in the spinal cord (Tsuda et al., 2003). A noticeable decrease in neuropathic

pain was observed in both P2X4 knock-down and knock-out mice (Ulmann et al., 2008, Tsuda et al., 2009). It was shown that mechanical hypersensitivity was developed after intrathecal injection of ATP-stimulated microglia in normal mice (Coull et al., 2005). This is consistent with the observations in studies, which revealed that using minocycline causes inhibition of activated microglia (Raghavendra et al., 2003, Narita et al., 2006, Hathway et al., 2009b, Pabreja et al., 2011). These studies consistently indicated that minocycline reduces microglial activation and mechanical allodynia in neuropathic pain models. Taken together, these observations underscore the critical role of P2X4 receptors in spinal microglia to mediate neuropathic pain.

1.5.5 Microglial activation, receptors and inducers for activation

Microglial immune responses are heterogeneous and exhibit region-dependent diversity (Hanisch, 2013). According to their functional activation, microglia respond differently depending on the types of stimuli (Colton and Wilcock, 2010). Microglial cells are usually stimulated by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Figure 1.1) (Block et al., 2007). Moreover, activation of microglial cells leads to gene expression activation of several genes that are involved in tissue repair such as chitinase 3-like and arginase1 (Arg1) (Colton, 2009).

The ability of microglia to respond and quickly restore homeostasis in injured tissue requires migration of the cells to the injury site to perform phagocytosis. To do this microglia express numerous surface receptors that facilitate the interaction with the stimulus such as LPS, ATP and HMGB1 (Figure 1.1). These microglial receptors and ion channels include inwardly-rectifying potassium (K^+) channels and other ion

channels (Kettenmann et al., 1993), purinergic receptors (Langosch et al., 1994), pattern recognition receptors (PRR), (Priller et al., 1995), receptor for advanced glycation end products (RAGE) (Ravichandran, 2003), neuro-hormone receptors, scavenger receptors, toll-like receptors (TLRs) (Landreth and Reed-Geaghan, 2009) and CX3CR1. Overall, signalling through these receptors cause alterations in membrane potential, intracellular Ca^{2+} concentration, cellular motility and cytokines production (Kettenmann et al., 2011). Increase of intracellular Ca^{2+} is also essential for microglial activation, including migration, proliferation, and release of NO, BDNF and pro-inflammatory cytokines (Kettenmann et al., 2011). Moreover, disturbance of microglial Ca^{2+} homeostasis causes activation of death programs, which are regulated by the microglia activation status.

1.5.5.1 High mobility group box 1 (HMGB1)

HMGB1 is a nuclear DNA-binding protein that is abundant in the nucleus of most mammalian cells and functions as a nuclear cofactor in transcription regulation (Ueda and Yoshida, 2010, Yanai et al., 2011). HMGB1 can be actively secreted from macrophages, monocytes and endothelial cells, or passively released by injured and necrotic cells to serve as a DAMPs to initiate inflammatory responses (Andersson and Tracey, 2011). In addition, HMGB1 can induce cytokine release and regulate the activity of different cells, including leucocytes, lymphocytes, glial cells and neurons (Yang and Tracey, 2010). HMGB1 mediates cytokine release, inflammation and tissue injury via several receptors, including TLR4, TLR2 and RAGE receptors (van Beijnum et al., 2008). Many studies revealed that the HMGB1 has been associated in the pathogenesis of several inflammatory disorders, including cancer, arthritis,

ischemia, epilepsy and chronic pain (Lotze and Tracey, 2005, Sims et al., 2009, Harris et al., 2012).

The passive release of HMGB1 by necrotic cells is rapid and increases cell permeability, whereas the active release is slow, and both mechanisms can produce the release of significant amounts of extracellular HMGB1 (Scaffidi et al., 2002, Gardella et al., 2002). HMGB1 orchestrates inflammatory responses, including cell migration stimulation, triggering of different immune cells responses and inhibition of phagocytosis (Oppenheim and Yang, 2005). The interaction between inflammatory mediators and HMGB1 may extend the inflammatory state which indicates that HMGB1 may prolong persistent pain states (Maeda et al., 2013).

In addition, many reports suggest that HMGB1 signalling in cortical cells may have a role in the development of neuropathic pain and mediates rapid changes in neuronal excitability (Shibasaki et al., 2010, Maroso et al., 2010). For example, Shibasaki et al. (2010) revealed that perisciatic administration of HMGB1 produces mechanical allodynia, whereas the thermal hyperalgesia can be partially reversed by treating the animal with anti-HMGB1 antibody in neuropathic pain models. Moreover, another study used tibial nerve injury (TNI) model to investigate the contribution of HMGB1 in neuropathic pain (Feldman et al., 2012), reported that TNI stimulates the re-distribution of HMGB1 in sensory neurons from nucleus to cytoplasm without altering its protein levels.

1.5.5.2 Adenosine tri-phosphate (ATP)

ATP is released in large amounts into the extracellular environment of injured cells. ATP is considered a key molecule in stimulating and maintaining reactive microglia in case of pathological conditions (Shigemoto-Mogami et al., 2001). In addition, ATP can cause microglial chemotaxis resulting in microglial accumulation in damaged areas (Honda et al., 2001). ATP activates microglia through ionotropic P2X receptors such as P2X7 and P2X4 and metabotropic P2Y receptors such as P2Y12 (Ferrari et al., 1996). Many cellular responses are induced in ATP-activated microglia including, increased intracellular Ca^{2+} concentration (Möller et al., 2000), phosphorylated mitogen-activated protein (MAP) kinase (Gao et al., 1999) and release of inflammatory cytokines such as IL-1β (Ferrari et al., 1996) and IL-6 (Shigemoto-Mogami et al., 2001). A recent study revealed that P2Y12 and THIK-1, which is the main K^+ channel expressed in microglia, are important for microglial ramification and surveillance of the brain. Once tissue damage happens, ATP is released which leads to the activation of P2Y12, potentiating the activity of THIK-1 and increasing intracellular Ca^{2+}, and thereby triggering release of IL-1β (Madry et al., 2017).

The cells respond differently to ATP depending on its concentration. For instance, a moderate ATP release has immune-depressive and anti-inflammatory effects (la Sala et al., 2001, Wilkin et al., 2002). In this case, ATP activates P2Y11 receptor that leads to increased secretion of IL-12 and the anti-inflammatory cytokines IL-4 and IL-10, while the pro-inflammatory chemokines are repressed (la Sala et al., 2003, Boeynaems and Communi, 2006, Bours et al., 2006). In contrast, a high concentration of ATP causes P2X7 receptor stimulation, which leads to massive release of pro-inflammatory mediators such as IL-1β and IL-18 (Di Virgilio, 1995, Surprenant et al., 1996).

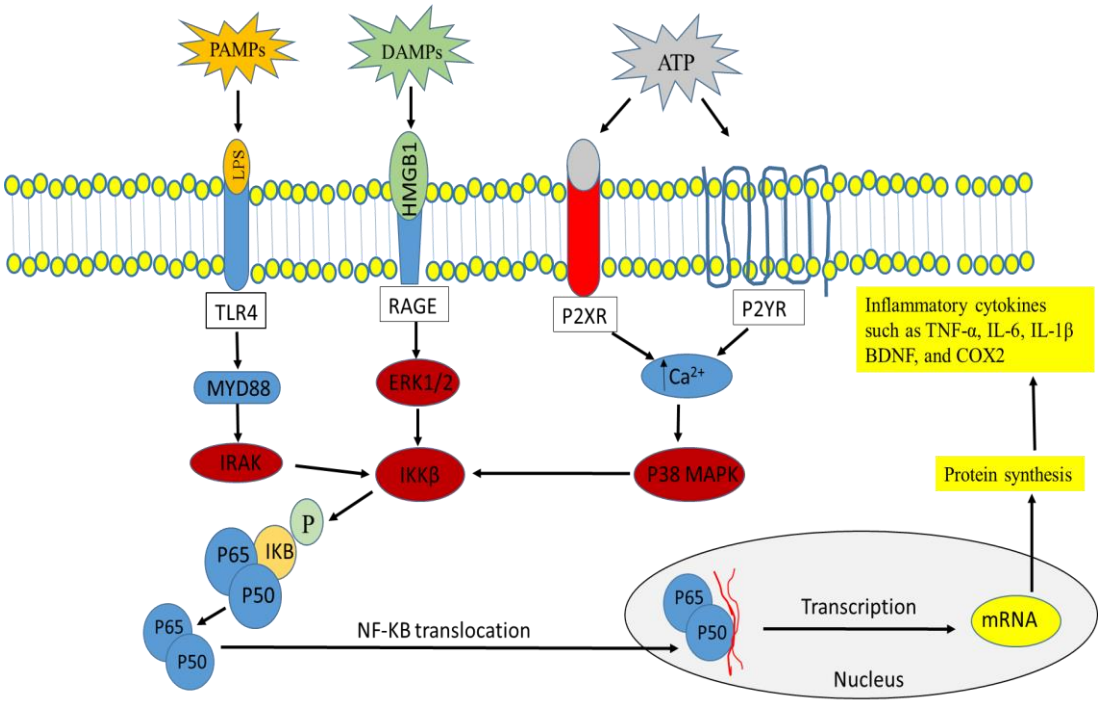


Figure 1.1. Schematic diagram of the microglia signalling pathways. Microglia express membrane receptors such as toll-like receptors (TLRs), receptor for advanced glycation end products (RAGE) and ATP receptors (P2X4, P2X7 and P2Y12). These receptors recognize pro-inflammatory stimuli such as pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) and cytokines. Ligand bound PAMPs and DAMPs activate downstream adapter proteins such as myeloid differentiation primary response protein 88 (MyD88), and MyD88 activates specific protein kinases such as mitogen activated protein kinases (MAPK) such as IRAK, TAK1, NIK and ERK1/2. These kinases activate IkB kinases (IKKβ) that phosphorylate IkB-α. In stimulated microglia, phosphorylation of IkB leads to its dissociation from the complex, and its proteasomeal degradation, allowing NF-kB to translocate to the nucleus, where it binds to specific DNA sequences present in the promoters of numerous target genes, Encoding the pro-inflammatory cytokines (IL-1, IL-2, IL-6, TNF-α), Chemokines (MIP-1a, MCP1), adhesion molecules (ICAM) as well as Cyclooxygenase-2 (Cox-2) and inducible nitric oxide synthase (iNOS).

1.5.6 Neuron-microglia and astrocyte-microglia crosstalk

Microglia-neuron interaction plays an essential role in the developing and damaged CNS (Kettenmann et al., 2011). Microglia both influence and are influenced by neurons and astrocytes (Figure 1.2) (Faustmann et al., 2003, Min et al., 2006, Kettenmann et al., 2011, Ousman and Kubes, 2012). *In vitro* models investigating co-cultured microglia with neurons or astrocytes have shown important insights about microglial phenotypes and functions (Claycomb et al., 2014, Dambach et al., 2014). Molet et al. (2016) demonstrated that the microglial Janus kinase/signal transduction and activator of transcription 3 (STAT3) activity effects the functional characteristics of spinal cord astrocytes and neurons by inducing mRNA expression and cell plasticity changes that are associated with neuropathic pain conditions.

Activated microglia produce IL-6, inducible nitric oxide synthase (iNOS) and TNF-α, resulting in production of ROS and NO resulted in neuronal death (Tanaka et al., 2013). Moreover, microglia are found surrounding α-synuclein-immunoreactive nigral aggregates and extracellular Lewy bodies, which resulted in enhanced α-synuclein-induced dopamine toxicity (Kim and Joh, 2006). Not only do stimulated microglia enhance neurotoxicity in pathological conditions, but they also release some cytokines and neurotrophic factors that modulate survival and repair neurons (Su et al., 2014). For example, damaged neurons release IL-4, which is involved in microglia polarization towards the anti-inflammatory phenotype (Podbielska et al., 2016). This is in concordance with observations demonstrating that the inhibition of microglial activation reduces neurotoxic effects and increases neuronal survival.

Fractalkine (CX3CL1) is a transmembrane glycoprotein which is mainly expressed in neurons, affecting the microglia by binding to its receptor CX3CR1 (Cook et al., 2001). Phagocytic mechanisms in microglia may occur through signalling of fractalkine to CX3CR1 receptors (Hughes et al., 2002) and $CX3CR1^{-/-}$ mice show a delay in synaptic pruning and dysregulation in microglia response in the developing brain (Cardona et al., 2006, Paolicelli et al., 2011). Moreover, the interaction between fractalkine and its receptor decreases microglial activation. Mizuno et al. (2003) reported that treatment of LPS/INF-γ stimulated microglia with fractalkine suppresses the release of TNF-α, IL-6 and NO and significantly reduces neuronal death.

Microglia and astrocytes are the main components of the CNS immune response (Liu et al., 2011). Their functions include the release of cytokines such as IL-6, IL-10, TNF-α, secretion of growth factors, and they also contribute in synaptic transmission modulation through removing neurotransmitters from the synaptic cleft such as glutamate (Lu et al., 2010, Sun et al., 2016). Astrocytes are in close contact with microglia, and the cross-talk between the two types of cells promotes the physiological and pathological responses (Figure 1.1) (Ni and Aschner, 2010). In addition, astrocytes express a variety of membrane receptors for neurotransmitters to control neuronal activity and to interact with signals from microglia (Ni et al., 2011). Astrocytes reduce microglial activation by other ways including the reduction of the production of molecules related to antigen presentation, up-regulation of tumour growth factor (TGF)-β and galectin-1 and inhibiting the production of pro-inflammatory mediators such as NO, TNF-α and ROS (Liu et al., 2011, Savarin et al., 2015).

The close contact of microglia and astrocytes may lead to time-dependent effects. For instance, IL-1β is mainly secreted by microglia and increases the neurotoxicity of stimulated astrocytes (Liu et al., 2011). In response to injury, ATP released from astrocytes stimulates the microglial cells, which prominently express purinergic receptors such as P2X7 (Hung et al., 2010). In addition, astrocytic Ca^{2+} waves play many physiological roles including, synaptic activity, controlling the microcirculation and maintaining ion homeostasis. These calcium waves spread between cells by gap junctions and may affect microglial cells (Hung et al., 2010, Liu et al., 2011).

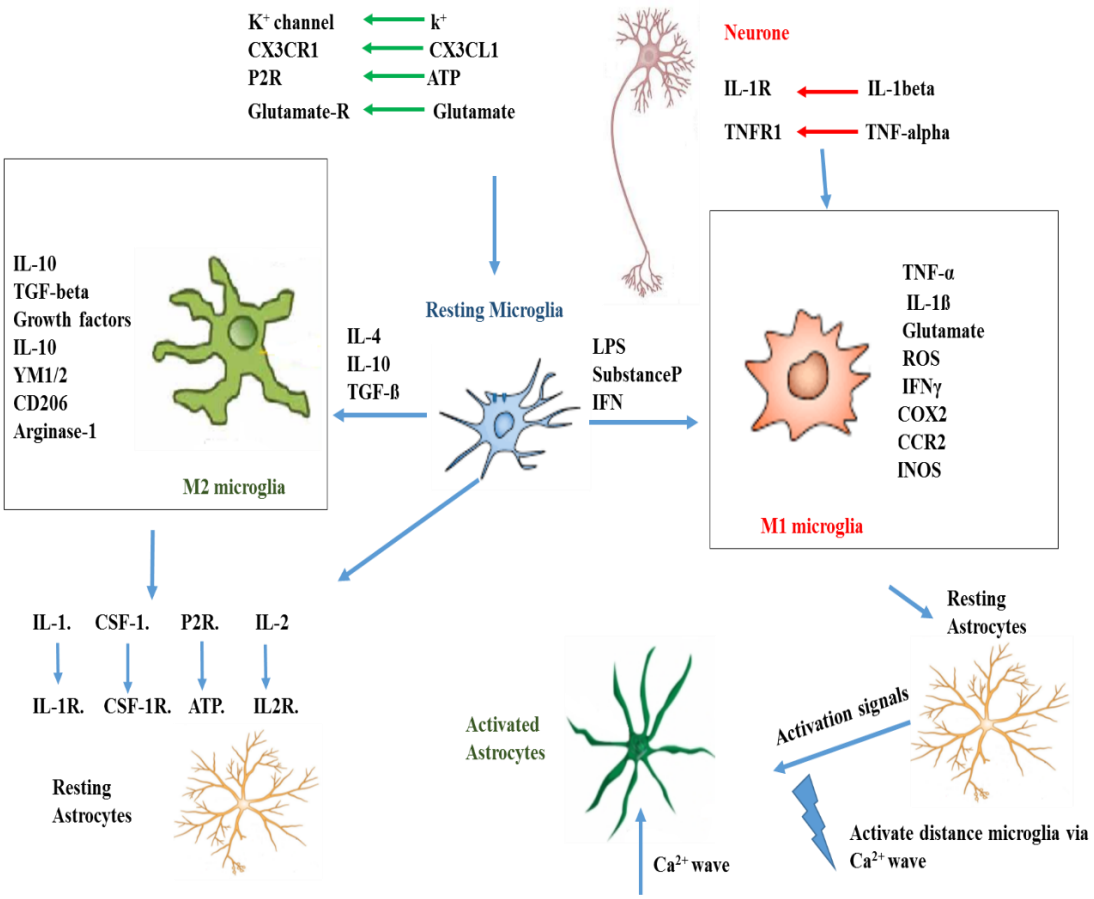


Figure 1.2. Schematic diagram showing the interaction of microglia with astrocytes and neurons in normal and pathological conditions. Neurons release immune-mediators including, Ca^{2+}, CX3CL1, TGF-β and ATP, which bind to receptors that are expressed in microglia, leading to an increase in both normal and pathophysiological processes. The activated microglia produce inflammatory cytokines, including IL-1β, TNF-α, and BDNF which trigger neurotoxicity through receptors that are expressed in neurons. In addition, the inflammatory mediators, including ROS, iNOS, TNF-α and IL-1β from activated microglia disturb the endothelial cells ability to preserve blood brain barrier (BBB) integrity, leading to a change in expression of critical molecules. Altered BBB permeability facilitates the penetration of tissue macrophages into the CNS. Anti-inflammatory mediators including IL-4, IL-10 and TGF-β change microglia towards anti-inflammatory phenotype, whereas LPS and IFN-γ stimulate microglia towards pro-inflammatory phenotype. Alternatively, astrocytes express receptors for the inflammatory mediators IL-1, CSF-1, and IL-2 that are mainly released by microglia. Stimulated astrocytes inhibit activated microglia through TGF-β, but activate distant ones through the calcium wave. Adapted from (Kabba et al., 2017).

1.5.7 Protective role of microglia

1.5.7.1 Anti-inflammation

IL-10, IL-4, TGF-β and IL-13 are the main anti-inflammatory cytokines that play a crucial role in reducing CNS inflammation and influencing the expression of genes that are associated with tissue repair (Colton and Wilcock, 2010). They also suppress the pro-inflammatory cytokines production such as TNF-α and IL-6, and decrease NO and superoxide release (Colton, 2009, Saijo and Glass, 2011). Neurons secrete IL-4 to enhance the expression of trophic factors in microglia (Zhao et al., 2015). IL-10 reduced microglial activation and repressed production of NO and TNF-α that resulted in neuroprotection against LPS-stimulated dopaminergic neuronal death in a mouse model of PD (Rentzos et al., 2009). TGF-β has many function including promotion of the extracellular matrix deposition, suppression of microglial activation by decreasing the expression of inflammatory factors including IL-1β, TNF-α and iNOS, and induction of angiogenesis which collectively participate in avoiding CNS damage after injury (Boche et al., 2006).

1.5.7.2 Trans-repression pathways

Accumulative evidence shows that the anti-inflammatory mechanisms of microglia may result from their regulation of trans-repression mechanisms through nuclear receptors. NF-$_k$B is the main effector downstream of TLRs and TNFα signalling pathway (Kawai and Akira, 2007, Hayden and Ghosh, 2014) and the molecular mechanisms of nuclear receptor affect this pathway. NF-$_k$B in its inactivated state is located in the cytoplasm bound to the inhibitory protein I$_k$Bα. Once the LPS bind to TLR4, the I$_k$B kinase is activated and phosphorylate the I$_k$Bα protein which result in the separation of I$_k$B from the NF-$_k$B complex. The activated NF-$_k$B then translocates

to the nucleus and recruits co-activators before subsequently binding to promoter regions of inflammatory genes (Figure 1.3) (Murphy and Crean, 2015, Le et al., 2016).

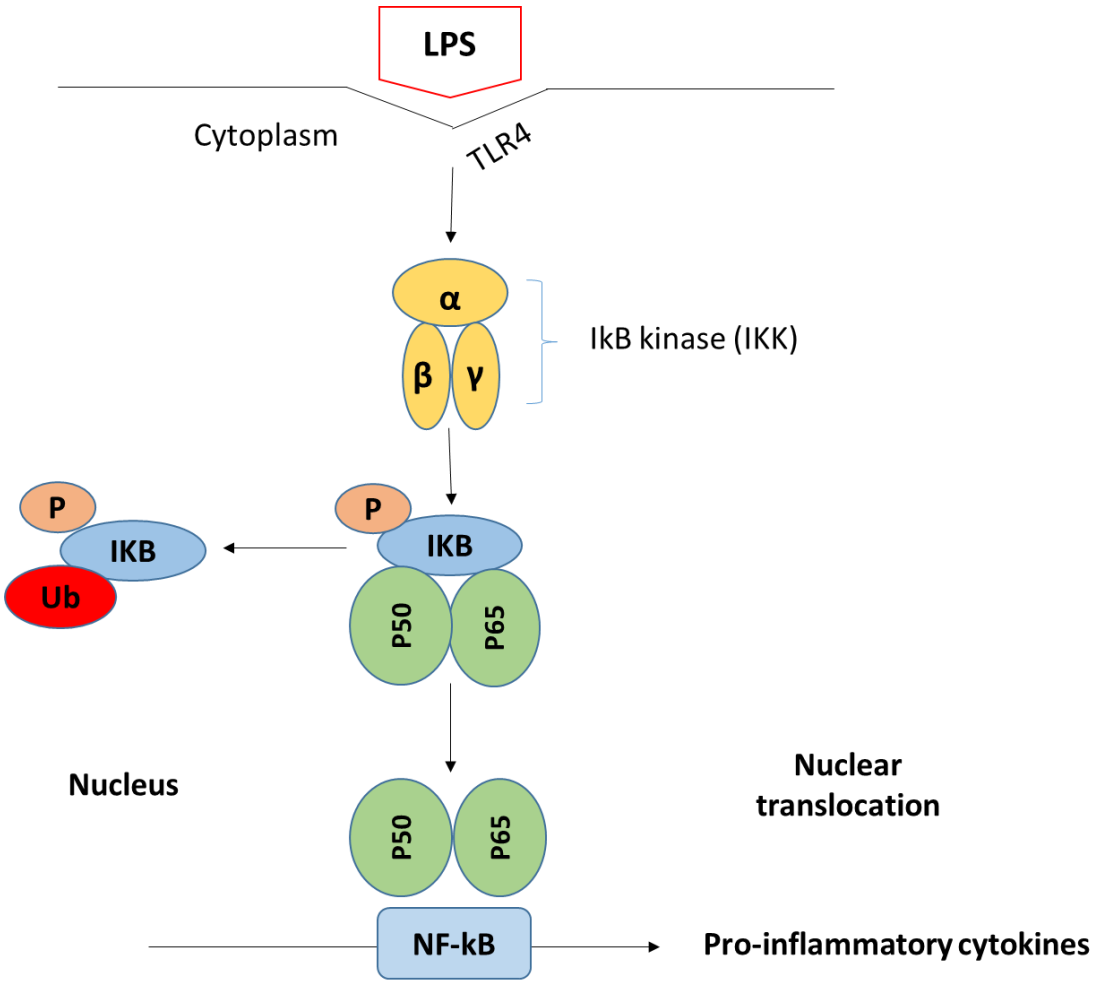


Figure 1.3. The NF-kB signalling pathway. NF-kB dimer p50/p65 is retained in the cytoplasm by the interaction with IkB kinase. The inflammatory activation results in the enzyme cascade consequently activating the IKK complex which phosphorylates IkB. Then, IkB is degraded by ubiquitin-proteasome system (UPS) pathway, this leads to release of NF-kB which subsequently enter the nucleus to induce target gene expression. Adapted from (Carrà et al., 2016).

A number of nuclear receptors have been described as having anti-inflammatory properties relevant to CNS inflammation. Glucocorticoids are potent anti-inflammatory and immunosuppressive agents detected by glucocorticoid receptors (GRs) and exert their anti-inflammatory effects through NF-$_k$B inhibition and down-regulation of a various of pro-inflammatory factors (D'Acquisto et al., 2002, Nelson et al., 2003). Deficiency of GRs stimulates microglial cells to produce NO and TNF-α which leads to dopaminergic neuronal death (Morale et al., 2004, Ros-Bernal et al., 2011).

Peroxisome proliferator-activated receptor (PPAR)s belong to the steroid hormone nuclear receptor superfamily, which plays a crucial role in neuroinflammation via trans-repression mechanisms (Dehmer et al., 2004, Quinn et al., 2008). The activation of PPARs by natural or synthetic agonists inhibits LPS-induced microglial activation through suppression of pro-inflammatory molecules expression such as TNF-α and cyclooxygenase-2 (cox-2) (Bernardo et al., 2000, Kim et al., 2002). PPARs activation in LPS-induced macrophage and microglia is also involved in repression of NF-$_k$B transcriptional activity by supressing the clearance of the NCoR/SMRT co-repressor complex (Figure 1.3) (Ghisletti et al., 2009, Xu et al., 2013). This NCoR/SMRT co-repressor interaction was also known as a main repressing mechanism for Liver X receptor (LXR), which is a nuclear receptor involved in inhibition of NF-$_k$B activity and cholesterol homeostasis as well (Ghisletti et al., 2009).

Emerging as a significant regulator of inflammatory processes and immune homeostasis in microglia through trans-repression mechanisms is the transcription factor nuclear receptor-related protein 1 (Nurr1/NR4A2). Nurr1 is a member of the

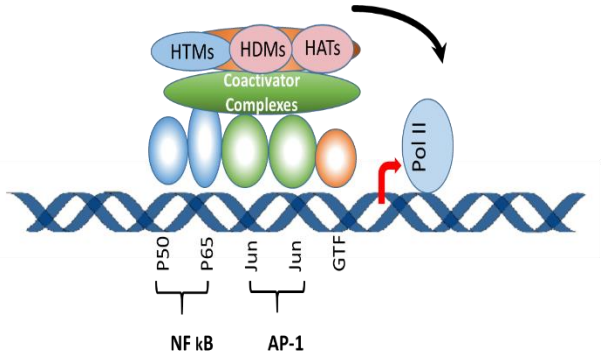
NR4A subfamily of orphan nuclear receptors, this group includes also Nur77 (NR4A1) and Nor1 (NR4A3) (Zhao and Bruemmer, 2010, McMorrow and Murphy, 2011). Nurr1 receptor activity is regulated via transcriptional regulation, post translational modifications, subcellular localisation and protein interactions (Zhao and Bruemmer, 2010). Subsequent studies revealed that Nurr1 regulates NF-$_k$B activity and inflammatory genes expression (Pei et al., 2005, Bonta et al., 2006, Saijo et al., 2009a).

In the LPS-injected PD model, Nurr1 acts in microglia and astrocytes to decrease the production of pro-inflammatory mediators that cause the death of dopaminergic neurons. Depletion of Nurr1 expression leads to amplification of pro-inflammatory responses in microglial cells (Saijo et al., 2009a). Saijo et al. (2009a) provided evidence for the mechanism by which Nurr1 mediates neuroprotection. The authors showed that Nurr1 is SUMOylated and phosphorylated which resulted in the recruitment of a Co-REST repressor complex at the promoter regions. Recruitment of this Co-REST complex helps in the clearance of NF-$_k$B /p65 from the promoter of inflammatory genes such as iNOS and reduction of inflammation (Figure 1.4). Moreover, Nurr1 association with the promoter regions of pro-inflammatory genes such as iNOS and TNF-α was also established in *in vivo* model. knockdown of Nurr1 by injection of lentiviral vectors expressing short-hairpin RNAs (shRNA) enhanced the hyper-inflammatory state in the cells, exhibiting an increase in iNOS and IL-1β production (Saijo et al., 2009a).

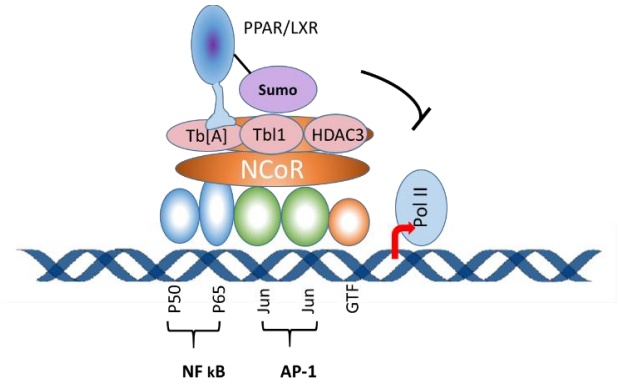
Like Nur77 and Nor1, Nurr1 acts the transcriptional activity by binding as a monomer, homodimer or heterodimer with retinoid X receptor (RXR) on cis-acting response

elements that are located in gene promoter regions (Aarnisalo et al., 2002, Ranhotra, 2015). The pharmacological modulation of Nurr1 activity can be achieved with Nurr1 agonist, including 1,1-bis (30-indolyl)-1-(p-chlorophenyl) methane (C-DIM12) and SA00025 which stabilise the association of CoREST/corepressor complex, inhibit pro-inflammatory gene expression by suppressing NF-$_k$B activity, and thus preventing dopaminergic neuronal death (Zhang et al., 2012a, Zhang et al., 2012b, De Miranda et al., 2015, Hammond et al., 2015). In addition, Nurr1 and forkhead box A2 (FOXA2), a potent Nurr1 co-activator in dopaminergic neurons, act synergistically to protect degenerating dopaminergic neurons by switching the microglia phenotypes (Oh et al., 2016). However, recent studies revealed that Nurr1 activity can be modulated through selective RXR-Nurr1 heterodimer agonists (RXR agonists), which act as a potential mono-therapeutic approach for PD (Spathis et al., 2017, Scheepstra et al., 2017). Collectively, the involvement of Nurr1 has been described in a range of cellular functions such as regulating neuronal survival, memory formation, neuroinflammation, hippocampal function and plasticity (Volakakis et al., 2010, Hawk and Abel, 2011, Hawk et al., 2012, Bridi and Abel, 2013). Accordingly, Nurr1 presents a promising target for therapy of neurodegenerative diseases.

A) TLR-dependent activation



B) PPAR/LXR trans-repression



C) Nurr1/CoREST trans-repression

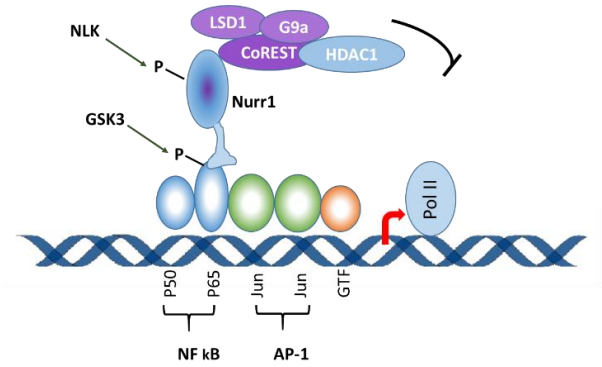


Figure 1.4. Nuclear receptors can alter NF-$_k$B signalling and activity. (A) Schematic diagram representing mechanisms by which nuclear receptors influence the NF-$_k$B signalling pathway downstream of TLR4. (B) LXR and PPAR repression mechanism of inflammatory response genes. Once the ligand bind to TLR4, LXR and PPAR are SUMOylated, and the Sumo targets both for interaction with NCoR complexes on targets promoter. This recruitment inhibits NCoR clearance in response to TLR signalling, which continue to supress gene expression. (C) Nurr1trans-repression mechanism of inflammatory genes. Nurr1 expression is induced by TLR4-mediated signalling and phosphorylates p65, which provides a binding site for Nurr1. Nemo like kinase (NLK)-mediated phosphorylation of Nurr1 causes the recruitment of the CoREST corepressor complex that inhibits NF-$_k$B target pro-inflammatory genes. Adapted from (Saijo et al., 2009a, Saijo et al., 2013).

Nurr1 transcriptional activity depends on many factors including, alternative splicing, post-translational modification, and the interactions with other nuclear receptors (Michelhaugh et al., 2005, Maxwell and Muscat, 2006, Sacchetti et al., 2006). The Nurr1 gene contains eight exons, and alternative splicing within exons three and seven potentially generates many variants as previously described in rat and human (Michelhaugh et al., 2005). However, studies comparing the relative abundance and transcriptional activity of the Nurr1 splice variants in glial cells are lacking. Like other nuclear hormone receptors, Nurr1 contains several functional domains including DNA binding domain (DBD), and carboxyl-terminal ligand binding domain (LBD). Two major regions critical for transcriptional activation, located in the amino-terminal activation function 1 (AF1) and a carboxyl-terminal activation function 2 (AF2) regions (Figure1.3.) (Castro et al., 1999, Chawla et al., 2001). The variants may be missing one of these regions and domains depends on the splicing sites. For instance, TINUR is a Nurr1 variant that formed through an alternative splicing event within exon three, resulting in shorter AF1 domain. Alternatively, splicing within exon seven produces Nurr1a, which produces a frame shift at the splice junction and forms a stop codon, shortening the carboxy-terminus (LBD and AF2) (Figure 1.3.).

SUMOylation of nuclear receptors has been described as playing crucial roles in trans-repression mechanisms (Pascual et al., 2005). The SUMO (small ubiquitin-like modifier) protein, is a 101-aminoacid polypeptide (Müller et al., 2001). There are four types of SUMO proteins 1, 2, 3 (Seeler and Dejean, 2003, Hay, 2005), and 4, which is only found in the human (Owerbach et al., 2005). The protein inhibitor of activated STAT (PIAS) family is a largest group of SUMO E3 ligases and consists of five isoforms (PIASxα, PIASxβ, PIAS1, PIAS3 and PIAS4) with specificity for either

SUMO isoform or substrate (Figure 1.5) (Kotaja et al., 2002, Palvimo, 2007). Knockdown of Ubc9 which is critical E2 ligase enzyme for SUMOylation pathway (Hay, 2005), reversed Nurr1-mediated suppression of iNOS, suggesting that SUMOylation is involved in Nurr1-mediated repression (Saijo et al., 2009b). Moreover, several studies reported that SUMO2 or SUMO3 using PIAS4 as an E3 ligase may be SUMOylated Nurr1 (Galleguillos et al., 2004, Saijo et al., 2009b). Mutational experiments showed that lysine 558 and 576 residues within LBD (Figure 1.6) are the SUMOylation sites of Nurr1 (Saijo et al., 2009b).

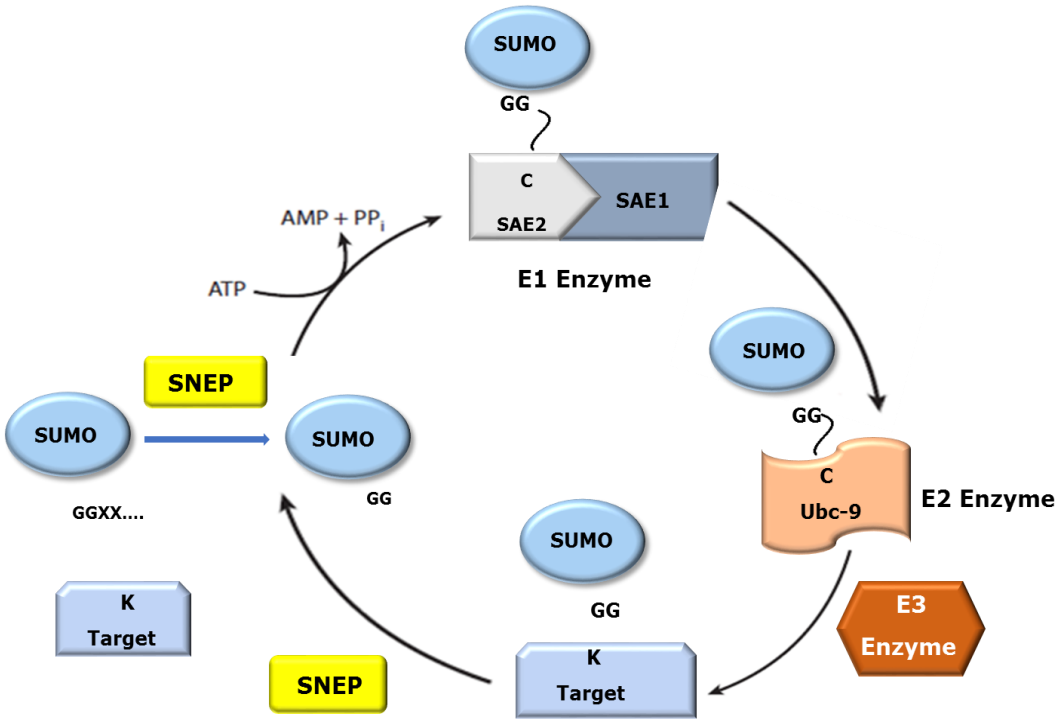
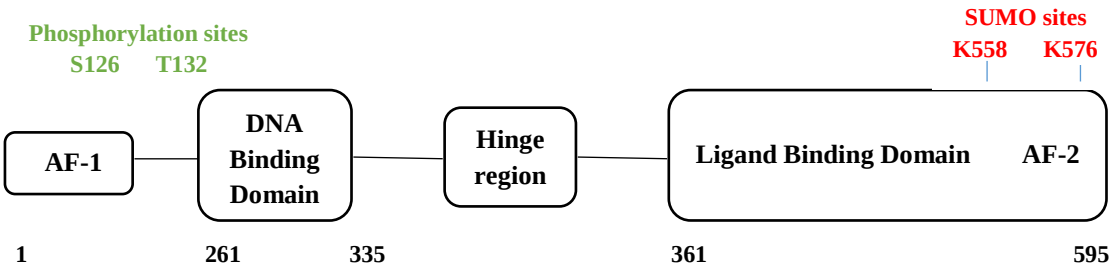


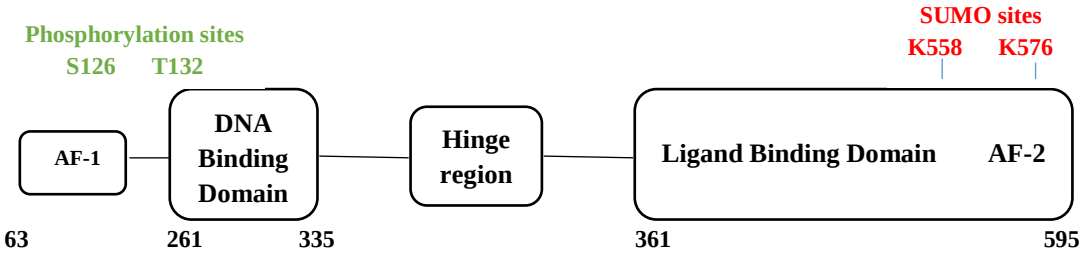
Figure 1.5. Schematic represent SUMOylation Pathway. SUMO is cleaved by SNEP enzymes. SUMO is activated by the SUMO-activating enzyme (SAE/E1), which is a heterodimer comprising SEA1/SEA2 in an ATP-consuming reaction. Activated SUMO protein is then transferred from E1 enzyme by conserved catalytic cysteine of the E2 enzyme (Ubc9) to lysine residue of the substrate, usually in conjunction with a specific SUMO E3 ligase. SUMOylation is reversible and this is accomplished by SUMO-specific proteases (SENPs) that cleave the iso-peptide bond and release SUMO for further cycles. Adapted from (Mukhopadhyay and Dasso, 2007).

Phosphorylation of Serine-468 residue in the NFκB subunit p65 is involved in negative regulation of NF-$_{k}$B signalling (Buss et al., 2004). Phosphorylation can be facilitated by glycogen synthase kinase (GSK)3β which is activated after TLR4 activation (Martin et al., 2005). However, inactivation of GSK3β enzyme using specific inhibitor (SB216763) leading to inhibit the interaction of Nurr1 and p65 in microglial cell line (BV2 cells) and stopped the recruitment of Nurr1 to the TNF-α promoter, resulting in increased transcription of TNF-α without altering the translocation of p65 to nucleus (Buss et al., 2004). These observations suggest that both SUMOylation of Nurr1 and phosphorylation of p65 are necessary for their interaction and subsequent trans-repression.

A)



B)



C)

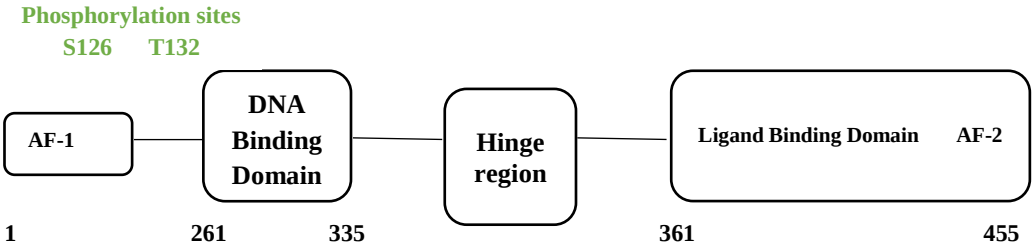


Figure 1.6. The schematic diagram shows rat Nurr1 isoforms domain structures and target sites. A) Full-length nurr1 has an activation function 1 (AF1) domain, a DNA-binding domain (DBD) and a ligand binding/activation function 2 (LBD/AF2) domain as indicated. ERK2 is a kinase which phosphorylates Nurr1 on multiple sites including S126 and T132, which are located near AF1 domain. Whereas lysine K558 and K576 are essential SUMO sites of Nurr1. B) TINUR isoform produced from alternative spicing within AF1 domain, and therefore missing 63 amino acids from the AF1 domain. C) Nurr1a isoform produced from alternative splicing within LBD/AF2, and therefore lacks 140 amino acids, which is mean that this isoform is missing the SUMOylation sites.

1.6 Developmental aspects of pain

In the last few years, the study of pain development in both humans and rodents has accelerated (Fitzgerald, 2005). Several studies indicated that the short- and long-term responses to noxious stimulus or inflammation change over postnatal development (Walker, 2013). The study of the developmental neurobiology of pain processing could contribute to understanding the early life behaviour and designing proper analgesics that are specific to young infants (Fitzgerald, 2005).

Descending inhibitory controls are immature in foetal period in both human and rodents, and the slow maturation of descending inhibitory pathways transferring from the brainstem through the dorsolateral funiculus of the spinal cord to the DH is mainly relevant to persistent pain in early period of life (Fitzgerald and Beggs, 2001). Hathway et al. (2009a) investigated the role of the RVM in the nociceptive processing in young rats. The study revealed that the RVM has an influence over spinal nociception, and this influence can shift from facilitatory modulation in the neonatal and adolescent period to biphasic facilitation and inhibition during the fourth postnatal week.

1.6.1.1 Role of microglia in neonatal pain

Neuropathic pain is not observed following early life peripheral nerve injury in both humans (Howard et al., 2014) and laboratory animals (Moss et al., 2007, Vega-Avelaira et al., 2012). This is partially due to developmentally regulated glial responses in the DH of the spinal cord. Developmental alterations occur in nociceptive pathways during the early postnatal period and the mechanisms of neuropathic pain in young animals are unlikely to be completely functional comapring to adults

(Fitzgerald, 2005, WALKER, 2008). The neuropathic pain model, spared nerve injury (SNI), in neonatal and young rat produced robust mechanical allodynia at 28 days of surgery, but animals aged 3, 10 and 21 days at the time of surgery did not show equivalent allodynia (Howard et al., 2005). Several studies have shown the role of spinal microglial activation in the pathogenesis of neuropathic pain following peripheral nerve injury (PNI) (Tsuda et al., 2005, Zhuang et al., 2005, Coull et al., 2005). Microglia are able to change from a surveillance role to activated state to mediate the neuropathic conditions (Coull et al., 2005, Tsuda et al., 2005).

The role of microglial cells in the development and maintenance of neuropathic pain in adults is recognised and controlled by inflammatory responses that result in neuronal sensitization in the DH (Gao and Ji, 2010, Taves et al., 2013). However, nerve injury in early life causes a little or no increase in the expression of either microglia (Iba-1, integrin-α) or astrocytes (GFAP) markers in the DH (Moss et al., 2007, Vega-Avelaira et al., 2007) and no increase in the pro-inflammatory cytokines expression such as TNF-α in the dorsal horn 7 days following injury (McKelvey et al., 2015). In the infant, nerve injury causes an increase in the expression of anti-inflammatory mediators such as GATA3 which is a transcription factor that regulates the secretion of anti-inflammatory cytokines such as IL-10 and IL-4, whereas the pro-inflammatory response is limited (McKelvey et al., 2015). However, the molecular regulation of pro- and anti-inflammatory microglial phenotype observed in the transition from the postnatal period to adulthood is not known.

1.6.2 Role of microglia in development

During embryogenesis, approximately 50% of the new-born neurons undergo programmed cell death and do not become mature (Schafer and Stevens, 2015). Microglia act as phagocytes for the removal of apoptotic cells (Ferrer et al., 1990, Peri and Nüsslein-Volhard, 2008, Sierra et al., 2010). In addition, microglia control the number of neural progenitor cells in the developing cerebral cortex by phagocytosing a proportion of viable cells with no apoptotic markers within the proliferative zone during cortical neurogenesis (Cunningham et al., 2013). Some findings indicated that CX3CR1 in microglia was essential for the survival of layer V cortical neurons in the postnatal brain and inactivation of microglia by minocycline treatment or transient ablation resulted in increased apoptosis in sub-cerebral neurons (Ueno et al., 2013). These findings highlight the biological significance of neuron-glia communication in network formation in the developing brain.

During CNS development, microglia interact with neuronal synapses, playing an important role in regulating synaptic function and maturation via elimination of synapses (Dalmau et al., 1998, Fiske and Brunjes, 2000). A deficit in synaptic pruning leads to a rather immature brain circuitry and is assumed to be involved in neurodevelopmental and neuropsychiatric disorders (Squarzoni et al., 2014, Zhan et al., 2014). Using immunocytochemical electron microscopy, Tremblay et al. (2010) were able to provide the structure and dynamic characterizations of the interactions between microglia and synapses under physiological conditions. They revealed that microglia behaviour changed during alterations in visual experience, such as increased phagocytosis of synaptic elements. Other studies have shown that microglia enhance neurogenesis and oligodendrogenesis by secreting specific combinations of IL-1β and

IL-6 into the sub-ventricular zone of developing rat brains (Ziv et al., 2006, Shigemoto-Mogami et al., 2014).

Disturbance of the microglial microenvironment through development may lead to improper brain functions, resulting in inappropriate expression of genes that are involved in inflammation, disturbing neuronal development, and triggering CNS disorders in the adulthood (Kettenmann et al., 2013). For instance, in models of early life stress, Chocyk et al. (2011) reported that maternal separation led to an increase in microglial caspases within the substantia nigra of juvenile male rats, which may have led to a decrease in the number and proliferation of microglia in this region in the adult. Additionally, maternal exposure to viral infection resulted in an increase in the risk of schizophrenia and autism in mouse and human offspring (Garbett et al., 2012).

1.7 Aims and hypotheses for chapter 4 and 5

The effect of PAMPs and DAMPs on microglia and the implication in inflammatory processes has been described in detail in chapter 1 (section 1.5.5). The identification of microglial regional heterogeneity in the neonates and adult rat CNS suggests possible regional differences in the sensitivity to different challenges. I hypothesise that microglial transcriptional profiles of inflammation are regionally heterogeneous and I postulated that ageing influences microglial inflammatory phenotype in a region-dependent manner. On the other hand, Nurr1 is expressed in dopaminergic neurons during life, suggesting a crucial role in development and maintenance of dopaminergic neurons (Yan et al., 2016). A growing number of studies have proposed that Nurr1 mediates transcription activation and plays a regulatory role in dopaminergic neuronal differentiation, preventing their loss by supressing the release of pro-inflammatory

mediators by microglia and astrocytes (Decressac et al., 2013, Yan et al., 2016). I hypothesise that Nurr1 mediates anti-inflammatory effects in microglia over the postnatal period.

Aims:

1) To investigate the global molecular basis for the potential phenotypic alteration of microglia over the postnatal period.

2) To illustrate the differential expression profiles of microglia from brain and spinal cord.

3) To determine the effect of PAMPs and DAMPs on the neonatal and adult microglial inflammatory transcriptome.

4) To investigate the anti-inflammatory roles of Nurr1 in neonatal and adult microglia.

1.8 Cannabinoids involved in pain modulation

Mammalian tissues express two types of cannabinoid receptors CB_1 and CB_2; both are G-protein coupled receptors (GPCRs) (Graham et al., 2008). The receptors are activated by many ligands including phytocannabinoids (from *Cannabis sativa*) such as Δ^9-tetrahydrocannabinol, synthetic cannabinoids and endogenous cannabinoids known as endocannabinoids such as 2-arachidonoylglycerol (2-AG) and anandamide (AEA) (Pertwee et al., 2010). The endocannabinoids are not stored in the cell prior to release but are thought to be synthesized on demand in the plasma membrane in response to elevation of intracellular calcium. The endocannabinoids have short duration of action due to effective enzymatic degradation (Hohmann et al., 2005).

AEA and 2-AG are derivatives of arachidonic acid conjugated with ethanolamine or glycerol, respectively. 2-AG is a full agonist at both CB_1 and CB_2 and it exhibits less affinity than AEA at both CB_1 and CB_2, whereas AEA shows slight selectivity for CB_1 over CB_2 receptors (Sugiura et al., 2000). AEA is the product of cleavage of the membrane phospholipid, *N*-arachidonoyl-phosphatidylethanolamine (NAPE) by the enzyme phospholipase D (PLD) that is found in different tissues including, brain, testis and kidneys (Okamoto et al., 2004). 2-AG production is associated with the metabolism of lipid (triacylglycerol) mainly by the activity of the enzyme diacylglycerol lipase (DAGL) (Stella et al., 1997). DAGL is located in the plasm membrane and its distribution is associated with the expression of 2-AG in tissues and organs (Carrier et al., 2004, Alexander and Kendall, 2007).

AEA and 2-AG act mainly through CB_1 and CB_2 receptors, which are both coupled to G_i/G_o proteins, thereby inhibiting adenylyl cyclase with subsequent reduction in

intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels. They also couple positively to mitogen-activated protein kinases and CB_1 receptors, via $G_{i/o}$ proteins, inhibit voltage-activated Ca^{2+} channels and activate inwardly rectifying K^+ channels (Rubovitch et al., 2002, FAN and YAZULLA, 2003). As with opioids, the overall effect of CB_1 receptor activation is to hyperpolarize neurons and reduce neurotransmitter release. The endocannabinoids function predominantly as retrograde messengers at GABAergic and glutamatergic synapses in the CNS; that is, they are synthesized post-synaptically by excitatory neurotransmitters (e.g. glutamate) that increase intracellular Ca^{2+} and diffuse back across the synapse to activate presynaptic CB_1 receptors, thereby inhibiting neurotransmitter release (Lutz, 2004).

Endocannabinoids are degraded mainly by two enzymatic systems: AEA by fatty acid amide hydrolase (FAAH) and 2-AG by monoacylglyceride lipase (MAGL) which is a serine hydrolase enzyme (Di Marzo and Petrosino, 2007). FAAH is a member of the serine-hydrolase family and it is distributed through the brain, including pyramidal cells in the cerebral cortex and hippocampus (McKinney and Cravatt, 2005). However, MAGL is widely distributed in the nerve terminals of brain neurons, particularly in axon terminals of granule cells (Gulyas et al., 2004). Other enzymes, ABHD6 (Marrs et al., 2010) and ABHD12 (Savinainen et al., 2012) also control the accumulation of 2-AG at cannabinoidergic synapses. A summary of the biosynthesis, release and degradation of AEA and 2-AG is illustrated in Figure 1.7.

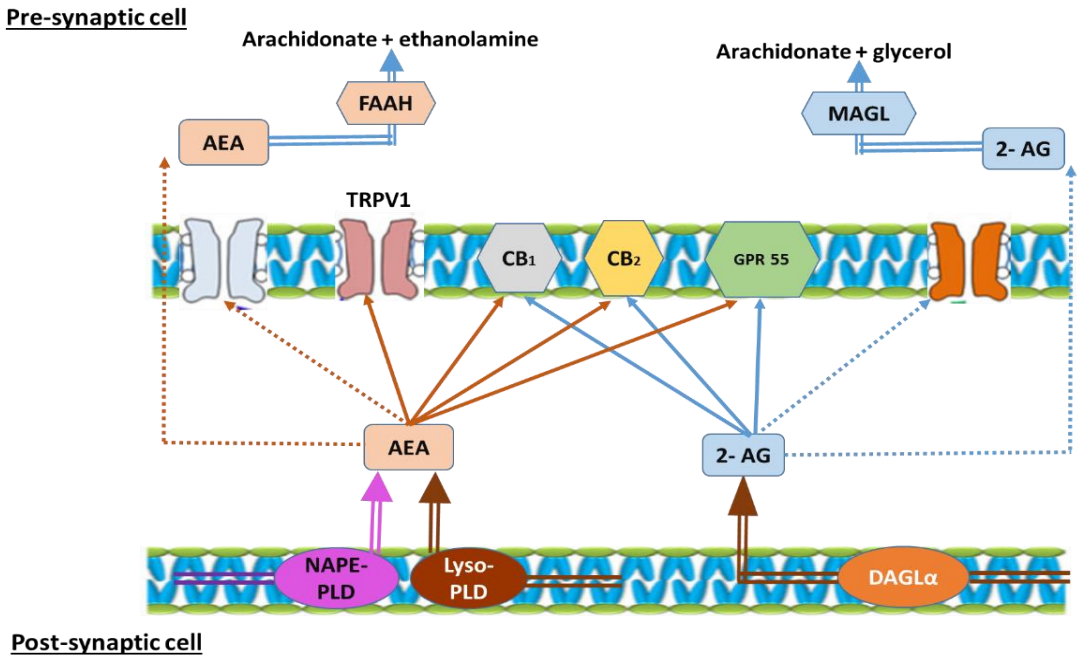


Figure 1.7. The major synthetic, signalling and catabolic pathways for anandamide (AEA) and 2-arachidonoylglycerol (2-AG). The synthesis, release and degradation of endocannabinoids. The AEA is synthesised from the precursor NAPE, which is released from membrane phospholipids, by NAPE-PLD. On the other hand, 2-AG is synthesised by DAGL through a phospholipase C (PLC)-dependent pathway. AEA and 2-AG are exposed to rapid degradation, anandamide is mainly degraded by the enzyme FAAH, whereas 2-AG is substrate for the enzyme MAGL. Adapted from (Burston et al., 2013).

CB_1 receptors are the most abundant GPCR in the CNS and are mainly expressed in cortical structures, including the hippocampus, amygdala and caudate putamen (De Fonseca et al., 2005). Furthermore, CB_1 immunoreactivity is found in the periaqueductal gray (PAG) and spinal trigeminal tract (Tsou et al., 1998a). CB_2 receptors have been demonstrated to exist mainly in immune cells such as T-lymphocytes, monocytes, polymorphonuclear neutrophils and microglia (Facci et al., 1995). However, many studies confirmed that CB_2 receptors are expressed in other tissues and organs including different regions throughout CNS such as the brain stem (Van Sickle et al., 2005), thalamus (Gong et al., 2006) and cerebellum (Ashton et al., 2006).

All of the components of the endocannabinoid system are established in regions that are involved in the transmission and modulation of pain. Several studies have shown the involvement of presynaptic CB_1 receptors in the analgesic effects of cannabinoids through inhibition of excitatory neurotransmitter release in spinal cord (Tognetto et al., 2001, Lever and Malcangio, 2002). Microinjection of cannabinoid receptor agonists such as WIN-55,212-2 and HU-210 into supraspinal sites, including RVM, PAG, dorsal nucleus raphe, amygdala and thalamus resulted in anti-nociception (Martin et al., 1999).

In recent years, many studies have reported that there are other cannabinoid receptors in the brain, in addition to CB_1 and CB_2 receptors (Di Marzo et al., 2000, Hájos et al., 2001). The orphan GPCR GPR55 is possibly one of the non-CB_1/CB_2 cannabinoid binding sites because of its ability to interact with a number of cannabinoid ligands,

but defining GPR55 as a cannabinoid receptor is still controversial (Ryberg et al., 2007, Ross, 2009).

Human GPR55 is 319 amino acids and, phylogenetically, it is different from CB_1 and CB_2 displays low amino acid similarity (13.5% and 14.4% respectively). GPR55 is structurally distinct from the classical cannabinoid receptors, due to its lacking of a cannabinoid binding pocket and because of the length of its third extracellular loop, that is significantly longer than the extracellular loop in the CB_1 and CB_2 receptors (Henstridge, 2011). GPR55 receptors are expressed in neurons and glial cells particularly in microglia, suggesting a possible role in neuro-immunological regulation (Pietr et al., 2009). Staton et al. (2008) showed that inflammatory mechanical hyperalgesia was absent in GPR55 knockout mouse models of inflammatory and neuropathic pain suggesting therapeutic potential for GPR55 antagonists.

1.8.1 Role of endocannabinoids in postnatal development

Several studies have revealed that the endocannabinoid system plays a role in brain development and maturation. Exposure to cannabinoids, such as marijuana preparations in the prenatal period causes modification of the maturation process of many neurotransmitter systems, including serotonin, opioids and dopamine (Fernández-Ruiz et al., 1999). CB_1 and CB_2 receptor mRNA is present at day 11 of gestation in foetal rat brain (Buckley et al., 1997) and at week 14 of gestation in the human embryo (Biegon and Kerman, 2001). The expression of CB_1 receptors increases progressively in the hippocampus, cerebellum, basal ganglia and frontal cortex in early life and adulthood (Mato et al., 2003). However, the expression of CB_2

receptors in postnatal brain and the role of this receptor over development remains unclear and needs more investigation.

Endocannabinoids are also found at an early stage of brain development. The AEA concentration is 1000-fold lower than that of 2-AG during the foetal period (Fernández-Ruiz et al., 2000). The developmental patterns of AEA and 2-AG are different and AEA concentration increases gradually throughout the foetal period until adulthood (Berrendero et al., 1999), whereas foetal levels of 2-AG parallel those observed in adult rat brain, and reach a maximum at birth (Berrendero et al., 1999).

The development of brain involves different processes including neurogenesis, neuronal migration and neuronal specification. The roles of CB_1 and CB_2 receptors in the regulation of neural proliferation and how they control cell fate remain poorly understood (Anavi-Goffer and Mulder, 2009). Pharmacologically, inhibition of CB_1 receptor function hinders proliferation in both developing and adult brain (Mulder et al., 2008). Treatment of neurons with CB_1 or CB_2 agonists enhances their proliferation rate *in vitro* (Aguado et al., 2005). Prenatal exposure to the phytocannabinoid Δ^9-THC increases the number of interneurons that express cholecystokin (CCK) which is a neuropeptide, which modulates neuronal migration (Giacobini et al., 2004). Autoradiography and immunohistochemistry studies showed that the distribution of CB_1 receptors on elongating axons in the corpus callosum between GD (14.5-18.5) in the rat brain and its expression are decreased when axons reach their final destination (Mulder et al., 2008); this indicates a probable role of CB_1 receptors in axonal guidance.

1.9 Aims and hypothesis for chapter 3

Previous studies have shown that pain behaviours are inappropriate during the early postnatal period (Slater et al., 2006), which reveals the immaturity of the pain circuitry (Andrews and Fitzgerald, 1994, Hathway et al., 2009a). Different studies have also revealed that endogenous cannabinoid signalling pathways undergo significant postnatal alteration in rodents (Piyanova et al., 2015, Kwok et al., 2017). We hypothesise that the endogenous cannabinoid systems undergoes significant postnatal changes within the human brain during postnatal period, and these changes are critical to the maturation of pain processing during this period. The aim of Chapter three is to elucidate the expression of the components of the endocannabinoid signalling system within the human midbrain and cerebellum during postnatal maturation.

2 Chapter Two: Materials and Methods

2.1 Subjects

Nottingham BioBank has kindly provided us with human tissues. Ethical approval for this study was sought and granted (Study ACP0000100). All experiments were conducted in accordance with The Human Tissue Act (2004) in registered laboratories. Selected tissues were obtained from those samples held within the biobank that had been donated with informed consent for its use in research. Samples included brain tissue that was donated by individuals aged 3-8 months post-birth and older than 14 years. Moreover, samples were additionally obtained from cases in gestational stage of 25-29 weeks and 38-39 weeks. These samples were available as paraffin embedded blocks which included mid-brain and cerebellum.

2.2 Animals

Postnatal day P1 and adult P40 Sprague Dawley rats (Charles River, UK) were used. Free accessibility of food and water was insured throughout all experiments. All procedures were done in P1 and P40 during the animals' light cycle. The experimental protocols are carried out in accordance with Home Office License 40/3647, the IASP guidelines and Animals (Scientific Procedures) Act 1986.

2.3 Drugs

LPS, poly-L-lysine, ATP and TRI reagent were obtained from Sigma-Aldrich (St. Louis, MO, USA). HMGB1 was acquired from HMGBiotech (Milano, Italy). Fractalkine was purchased from R&D systems (Minneapolis, USA). All drugs were prepared as stock solutions in the distilled water except fractalkine which was prepared in PBS and kept at -20˚C. Dilutions in the culture media were prepared from stock

solutions before application. No detectable effect of solvents was found in the experiments.

2.4 Primary microglial isolation and culturing

2.4.1 Primary microglia isolation from neonatal rat brain and spinal cord

Primary microglia were isolated from postnatal day P1 to P2 rat pups as described by (Tamashiro et al., 2012). The neonatal rat pups were rinsed with 70% ethanol, then decapitated with sterile sharp scissors. The head and the whole body were immediately dropped into 70% ethanol, and then transferred into saline. The whole brains and spinal cords were removed and placed into petri dishes with chilled 5ml L-15 solution (Leibovitz's L-15 conditioned media, 0.1%BSA, 1.0% Penicillin/streptomycin). The meninges were removed from the brains and spinal cords which were then transferred into a new petri dish filled with 5ml of L-15 conditioned media. The tissues were then transferred with a 10ml pipette to a sterile 50ml tube and centrifuged at 2,500xg for 5min at 4˚C (Beckman Coulter, USA). The supernatant was aspirated and 5ml of fresh L-15 media was added. A single cell suspension of the brain and spinal cord tissues was prepared by gentle pipetting up and down with 10ml pipette and filtrated through a 100µm cell strainer in order to insure complete remove tissue debris. Consequently, the cell suspension was centrifuged at 2,500xg for 5min at 4˚C, followed by aspirating the supernatant and re-suspending the pelleted cells in 6ml of DMEM media, including 10%FBS and 1% penicillin/streptomycin. An equal volume of the cell suspension was transferred to each T-75 flask containing 12ml culture media (1 flask for each rat pup brain, or 1 flask for 3 spinal cords processed). The flasks were placed in a CO_2 incubator (Thermo, UK) at 37˚C to allow the cells to attach on the surface of flasks. The medium

was changed on the fifth day, and then every three days afterward and the mixed glial culture allowed to grow until they achieved confluence. Confluent 10 to 14-day old mixed glial cultures were then incubated on a shaker at 100rpm for 1-2 h in CO_2 incubator to promote the detachment of the microglia from the surface of the flasks. Immediately after shaking, the supernatants containing detached microglia were collected and centrifuged at 2,500xg for 5min at 4˚C and re-suspended in 1ml of fresh microglia media (DMEM, 10% FES, 1% penicillin/streptomycin). The primary microglia were counted using an automated cell counter (Innovatis, Germany) and seeded.

2.4.2 Primary microglia isolation from adult rat brain and spinal cord

Sodium pentobarbital was used to anesthetize 40-day old male rats via intraperitoneal injection and trans-cardially perfused via the left ventricle with saline solution supplemented with 2units/ml heparin (Wockhardt, UK) at an approximate hydrostatic pressure of 1ml water to remove blood cells from the brain and spinal cord. After 5min of perfusion, the brain and spinal cord were aseptically removed and carefully dissected free of meninges. The brains and spinal cords were cut into 500µm thick sections using sterile scalpel and transferred into 6ml Hibernate A media (Gibco, USA) containing 130U papain (Worthington, USA), and then incubated at 37˚C for 15min with gentle rotation. The tissues were removed from the incubator, gently triturated and returned to the incubator for a further 15min. Tissue dissociation was terminated by adding an equal volume of DMEM media and the cell suspension was passed through a 70µm cell strainer. Cells were then centrifuged at 397xg for 5min at RT, the supernatant was aspirated and pellet re-suspended in 20ml DMEM media. The cell suspension was transferred to tissue culture flask and incubated overnight in a

CO_2 incubator at 37˚C. The following day, the flasks were tapped to remove non-adherent cells, and the flasks were then washed twice with 15ml microglia culture media (DMEM, 10%FBS, 1% Penicillin/Streptomycin) was added to each flask. Microglia were maintained in this media for up 1 week, once the microglia were beginning to extend processes cells were utilized in experiments (Yip et al., 2009; Rustenhoven et al., 2016).

2.4.3 Culture and treatment of primary microglia

The neonatal and adult microglial cells were plated at a density 1.0 x 10^5 cells/ml of medium onto 12-well plates (for stimulation and NO production assay), or 24-well plates containing glass cover slips pre-coated with poly-L-lysine (for immunocytochemistry and phagocytosis assay) and incubated at 37˚C in a CO_2 incubator, and were used for experiments 24hrs after plating to allow microglia to attach onto the plates or cover slips. To induce inflammation, microglia cultured from neonatal and adult tissue were incubated with 100ng/ml LPS for 6hrs, 100µM ATP for 12 hrs, 500ng/ml HMGB1 for 12hrs or 5ng/ml Fractalkine for 24hrs prior LPS treatment. Cell viability was checked routinely throughout the culture period.

2.5 Immunocytochemistry

4% paraformaldehyde (PFA) was used to fix microglial cells for 10min followed by 3 washes of PBS. To permeabilise the cells 0.1% Triton X-100 in PBS was added for 10min. Then cells were washed with PBS twice for 5min each. Non-specific binding was blocked by 0.5% bovine serum albumin (BSA) in PBS for 1hr at RT. Cells were incubated in primary antibodies diluted in 0.5% BSA in PBS at 4˚C overnight. Cells were washed 3 times with PBS and incubated with appropriate anti-species secondary

antibodies diluted in 0.5% BSA in PBS for 45min at RT. Nuclei were stained with DAPI (10µg/ml) for 15min at RT and washed with PBS. Images were acquired using DMRB Leica fluorescence microscope. The antibodies that used in immunocytochemistry are summarized in Table 2.2.

2.6 Microglial phagocytosis assay

Microglia were incubated for 24hrs in culture medium containing 100ng/ml LPS (Sigma, USA), followed by 3 washes with DMEM. Fluorescent latex beads (1µm diameter, Sigma) were diluted 1:2000 in DMEM culture media (0.5µl beads per ml). 1 ml of diluted beads was added to each well and incubated for 2hrs at 37˚C in a CO_2 incubator. After washing 3 times with DMEM, cells were fixed for 15min using 4% PFA in PBS. Then the procedure was continued as previously mentioned in immunocytochemistry section. Fluorescent intensity of microglial cells was indicative of the amount of beads phagocytosed.

2.7 Microglial nitric oxide detection

Nitric oxide production in Microglial cells was assessed indirectly by quantification of nitrite in the culture media using the Griess reagent (Sigma). Microglial cells were plated in poly-L-lysine coated 12-well plates at $1.0x10^5$ cells per well. After attachment, microglia were treated with LPS (100ng/ml) for 6hrs, with ATP (100µM) for 12hrs or with HMGB1 (500ng/ml) for 12hrs. Following treatment, 500µl of the supernatant were collected from each well and an equal volume of the Griess reagent was added. The mixtures were incubated at RT for 30min. The absorbance at 546nm was measured using a microplate reader (BMG labtech, UK) and the nitrite concentration determined using a sodium nitrite standard curve.

2.8 Total RNA extraction and RNA quality assessment

2.8.1 RNA extraction from human tissue

10µm thick sections were sliced from each tissue block and placed with clean forceps into a sterile, RNase-free 1.5ml micro-centrifuge tube. The microtome (Leica 4060, Germany) and blade were cleaned with xylene between each block to prevent sample-to-sample contamination. The total RNA was purified using the FFPE total RNA isolation kit (Invitrogen, USA) according to manufacturer's instructions with slight modification as following: 800µl of melting buffer was added, to melt the paraffin, collecting the sections into tubes that were centrifuged briefly at 17,000xg to submerge the sections in melting buffer and incubated for 20min at 72˚C with gentle mixing every 5min. After that, 50µl proteinase K was added to the tube containing the tissue and mixed by pipetting up and down, then incubated for 60min at 60˚C. Immediately, tubes were centrifuged at 17,000xg for 1min to separate the tissue lysate from paraffin, which formed a thin layer at the top. The solution containing the tissue lysate was aspirated and transferred to a clean tube. To isolate the total RNA, 400µl binding buffer was added with 800µl 100% ethanol to the tissue lysate and mixed by vortexing. 700µl of the sample above was transferred to the spin cartridge and spinned at 800xg for 1min, followed by discarding the flow through. The previous step was repeated with the remaining sample. The cartridge was washed with 500µl wash buffer three times; after each wash the cartridge was centrifuged at 17,000xg and the flow through discarded. The total RNA was eluted in 50µl of pre-heated RNase-free water by centrifugation at 17,000xg for 1min. The RNA was stored at -80˚C until further use. The quantity of the total RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo scientific, UK). To check the quality of RNA, the Agilent bioanalyzer application was used, which that indeed showed that the isolated RNA

from FFPE was degraded. All samples had an integrity value of less than 5 (RIN) (RIN= 28S to 18S rRNA ratio) which is considered not an acceptable value.

2.8.2 Total RNA extraction from microglial cells

Total RNA was extracted from primary microglial cells using Tri-Reagent and 1-Bromo-3-Chloro-propane (BCP) (Sigma, USA) as per the manufacturer's instructions as follows: cells were re-suspended in 300µl of TRI reagent and mixed thoroughly. 60µl BCP was added to cell lysate and the mixture was mixed thoroughly. This was separated into two phases by centrifuging at 10,000xg for 15min at 4°C, then the aqueous phase was taken to a new tube (the organic phase was stored for protein extraction). The RNA was precipitated by adding 0.2ml isopropanol. After thorough mixing, the final solution was incubated overnight at -20°C. The precipitated RNA was collect by centrifugation at 13,000xg for 10min at 4°C. The pellet was washed twice with 70% ethanol and centrifuged again. Any remaining alcohol was removed with a disposable pipette tip. The tube was stored open on the bench for few minutes to allow ethanol to evaporate. 87.5µl of DEPC-treated H_2O were added and the RNA extraction heated for 5min at 65°C. To remove any DNA contamination, 2.5µl of DNase I (10U/µl) and 10.0µl of RDD buffer were mixed and added to the RNA, and the solution incubated at RT for 10min. Total volume was made up to 400µl with DEPC-treated H_2O and the RNA was extracted with 400µl of phenol/chloroform/isoamyl alcohol, the mixture was mixed well then centrifuged at 10,000xg for 10min at 4°C. Upper aqueous phase that contains the RNA was removed to a fresh tube. Pure RNA was precipitated by adding of one tenth of the volume (40µl) of 3M sodium acetate, pH 5.2 and 2 volumes (800µl) of absolute ethanol. The mixture was incubated for overnight at -20°C. RNA was pelleted by centrifugation at 17,000xg

for 10min, then the RNA was washed once in 70% ethanol. Tube was stored opened on the bench for few minutes to allow the ethanol to evaporate. RNA was dissolved in approximately 40µl DEPC-treated water and stored at -20°C. The quality and quantity of the total RNA was assessed using a NanoDrop 2000 spectrophotometer.

2.9 Taqman quantitative real-time polymerase chain reaction (RT-PCR)

2.9.1 cDNA synthesis

250ng of total RNA was reversed transcribed as follows: the RNA was incubated with 300ng random primer at 65˚C for 5min. The mixture was cooled to allow primers to anneal to template RNA for 10 min at RT, and the following components were added to the mixture: 0.8µl dNTP mix (25mM), 2µl of 10x affinityScript RT buffer, 2µl of 100mM DTT, 0.5µl recombinant RNase inhibitor and 1µl reverse transcriptase. The total volume of the reaction was 20µl. The mixture was incubated at 25˚C for 25min, and then incubated at 50˚C for 60min. The reaction was terminated at 70˚C for 15min and cDNA stored at -20˚C for subsequent use as template in RT-PCR reaction.

2.9.2 Pre-amplification

To improve the quality of RNA that was extracted from the FFPE tissue and to enhance the analysis of the tissue samples, a pre-amplification step was carried out using Taqman master mix (Li et al., 2008). The primer pool mix was prepared by taking 5µl from each primer (10pM) of reference and target genes and diluting the pool using 1x TE buffer to a final concentration 0.2pM. The 25µl PCR reaction included 12.5µl of 2x Taqman master mix, 6.25µl of primer pool and 6.25µl cDNA. The reactions were incubated in PCR tubes at 95˚C for 10 min, following by 16 cycles of 95˚C for 15sec and 60˚C for 4min.

2.9.3 Taqman Primers and probes

Primers and probes were designed using Primer Express 3 software (Applied Biosystems, UK) and synthesized by Eurofins. Fluorogenic probes were labelled with FAM (6-carboxy fluorescein) or MGB (minor groove binder) at the 5' end and with quencher, TAMRA (6-carboxy-tetramthyl- rhodamine) at the 3' end. Sequences of primers and probes used for PCR studies are shown in Table 2.1.

Table 2.1 Sequences of primers and probes used in RT-PCR

Gene	Sequences 5' to 3'
Human's primers and probes:	
HMBS	Forward: CCAGCTCCCTGCGAAGAG Reverse: TTCCCCGAATACTCCTGAACTC Probe: CCCAGCTGCAGAGAAAGTTCCCGC
Cyclophilin A	Forward: CAAATGCTGGACCCAACACA Reverse: TGCCATCCAACCACTCAGTCT Probe: TGGTTCCCAGTTTTTCATCTGCACTGC
CB_1	Forward: AGAAAGCAGGCGCCCTAAC Reverse: TGATTAGGCTGAGCTCAAAATGACT Probe: ATTGCCCCCTGTGGGT
CB_2	Forward: AACACAACCCAAAGCCTTCTAGA Reverse: GTCACCCAGCATTCCTCCAT Probe: CTCAGTGGAATCTGAAGGG
FAAH	Forward: GCTGGCCTTCCTGGTGAA Reverse: TTCCAGCCGAACGAGACTTC Probe: CCTCTGCTGCCAAGGCTGTCAGC
NABE-PLD	Forward: CCAAACAAAGAAATCTATGGCAATT Reverse: TCTCTAGAGCTTCATTCAGCTTCACT Probe: AGCAAATGAGCATTACTT
GPR55	Forward: AATGACATCTCTCAGCCCTCTCA Reverse: GTCCCCACTGGTGTTTTGCT Probe: CTGCACCGGACCACCAACAGATGAGT
DAGL-Ɑ	Forward: TGGGCCACAAGGGTATGGT Reverse: CCACAATCAGGCCGTAGTGTT Probe: CCCAGGCCTTTGGGCGAGACC
Rat's primers and probes:	
GAPDH	Forward: TCTGCTCCTCCCTGTTCTAGAGA Reverse: CGACCTTCACCATCTTGTCTATGA Probe: ATCTTCTTGTGCAGTGCCAGCCTCGT
IL-1β	Forward: CACCTCTCAAGCAGAGCACAG Reverse: GGGTTCCATGGTGAAGTCAAC Probe: TGTCCCGACCATTGCTGTTTCCTAGG
IL-10	Forward: CCCTGGGAGAGAAGCTGAAGA Reverse: CACTGCCTTGCTTTTATTCTCACA Probe: CAGCTGCGACGCTGTCATCGATTTC
iNOS	Forward: CCACGCCAAGAACGTGTTC Reverse: GCCCTCGAAGGTGAGTTGAA Probe: CAGAGTGAGAAGTCCAGCCGCACCA
CX3CR1	Forward: GGGTGAGTGGCTGGCACTT Reverse: GATCCAATTCCGGGAAGGA Probe: CGTCCCCAGCTGCTCAGGACCTC
Sall1	Forward: AGTCGCCCCACTAAGAGCAA Reverse: GGAGCAGAAGGTCTGATAATTCAAA Probe: CCCACGTCTGTGGCCGGTGC
Nurr1	Forward: TTGCAGAATATGAACATCGACATTT Reverse: CCCGTGTCTCTCTGTGACCAT Probe: TCTCCTGCATTGCTGCCCTGGC
Nur77	Forward: TGTTGCTAGAGTCCGCCTTTC Reverse: CAGGCCTGAGCAGAAGATGAG Probe: TTATCCTCCGCCTGGCCTACCGATCTAA
Nor1	Forward: CGCCCTTGTCCGAGCTTTA Reverse: CGGTGGGACAGTATCTGGAGTAA Probe: CAGACGCAACGCCCAGAGACCTTG
TINUR	Forward: TCCACCTTTAATTTCCTCGAAAAC Reverse: CGTCGTAGCCTGTGCTGTAGTT Probe: CACTCGGCTGAAGCT
Nurr1a	Forward: AGCATACAGAATATGAACATCGACATT Reverse: CCCGTGTCTCTCTGTGACCAT Probe: CCTTCTCCTGCATTGCTGCCCTGT

2.9.4 Taqman RT-PCR

Gene expression was quantified by Taqman quantitative RT-PCR using the real time PCR system (Step One Plus Applied Biosystems, UK or Agilent, UK). 3µl of cDNA was added to a mixture of primers, probe, Taqman mix and DEPC H_2O to make up a total reaction volume of 13µl. A relative standard curve and negative template control were included every real-time PCR was run. A pool of all the sample cDNA was prepared, with a standard curve over four-fold dilutions. Efficiencies of the real-time PCR reactions ranged between 90%-100% and r^2 values were between 0.99-1.00. Measurements were carried out in triplicate and relative quantities assessed from the standard curve. mRNA expression levels of CB_1 and CB_2 receptors and the enzymes FAAH and NAPE-PLD were quantified in Human mid-brain and cerebellum, and the expression levels were normalized to the geometric mean of two reference genes; that did not change expression with development (Wong et al., 2009, Long et al., 2012), hydroxymethylbilane synthase (HMBS) and cyclophilin A. Moreover, there were no developmentally related changes in the expression of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in microglia. Therefore, mRNA expression levels of IL-1β, IL-10, iNOS, CX3CR1, Sall1, Nurr1, Nur77, Nor1, TINUR and Nurr1a were normalized to the GAPDH.

2.10 Protein preparation and Western blotting

2.10.1 Protein extraction from microglial cells

The protein from primary microglial cells was extracted using organic phase after RNA isolation. To precipitate DNA, 90µl were added of 100% ethanol to the mixture. The mixture was inverted and allowed to stand for 3min at RT, then centrifuged at 2,000×g for 5min at 4°C. The supernatant was transferred to new tubes for protein

isolation. Protein was precipitated from the supernatant by adding 0.5ml of isopropanol. Samples were allowed to stand for at least 10min at RT, then spinned at 12,000×g for 10min at 4°C. The supernatant was aspirated and the pellet was washed 3 times in 600µl 0.3M guanidine hydrochloride/95% ethanol solution. During each wash, samples were incubated in wash solution for 20min at RT, and then centrifuged at 7,500×g for 5min at 4°C. The protein pellet was air dried for 5min, then dissolved in 2.0% SDS. Insoluble material was removed by centrifugation at 10,000×g for 10min at 4°C. The supernatant was then transferred to a new tube. The protein was immediately used for Western blotting or stored at –20 °C.

2.10.2 Western blotting

Western blotting was performed using standard procedures. The protein were denatured and allowed to separate in 10% polyacrylamide gel. Subsequently, transferring the protein to nitrocellulose membrane was performed for 1h. The membrane was washed with TBS containing 0.1% Tween 20 (TBST), and the protein transferring step checked by adding a few drops of Ponceaus S solution, then washed with TBST. The remaining binding sites on the membrane were blocked for 60min incubation in TBST containing 5% non-fat dried milk at RT. Following the blocking, the membrane was incubated with primary antibodies in TBST containing 5% non-fat dried milk overnight at 4°C. Following washing with TBST, the membrane was incubated in secondary antibodies in TBST containing 5% non-fat dried milk for 60min at RT. The nitrocellulose membrane was washed with TBST. Finally, the immune complex was imagined using a Li-cor Odyssey scanner. The primary and secondary antibodies are summarized in Table 2.2.

Table 2.2 Primary and secondary antibodies that used in immunocytochemistry and Western blotting.

Antibodies	Dilution	Source
Antibodies used in Immunocytochemistry:		
rabbit anti-Iba-1 (microglial marker)	1:500	Wako (Lot# 019-19741)
rabbit anti-glial fibrillary acidic protein (GFAP) (astrocyte marker)	1:1000	Abcam (Lot# ab48050)
mouse anti-NeuN (neuronal marker)	1:100	Temecula (Lot# 2453249)
mouse anti-CC1 (oligodendrocyte marker)	1:500	Calbiochem(Lot#D001762 44)
Goat anti-rabbit (secondary antibody)	1:1000	Invitrogen (Lot# 54255A)
Goat anti-mouse (secondary antibody)	1:1000	Life Technology (Lot# 1696425)
Antibodies used in Western blotting:		
Mouse anti-Nurr1	1:1000	Abcam (ab41917)
Rabbit anti-β-actin	1:5000	Cell Signalling (4970)
donkey anti-rabbit (secondary antibody)	1:5000	Li-cor (C70308-05)
goat anti-mouse (secondary antibody)	1:5000	Li-cor (C00816-02)

2.11 Cloning

2.11.1 Primers design for cloning

The primers of the human CB_1 and CB_2 , rat Nurr1 and rat IL-10 promoter were designed according to data base sequences that were retrieved from NCBI (accession no. XM_006715330, XM_011540629, XM_017591997 and XM_006249712 respectively) (Table 2.3). A sequence of 1000 to 1500 nucleotides was targeted for amplification. The specificity of primers was tested by blasting them in the NCBI data base, and then ordered from Eurofins, UK. Once the primers were delivered, they were re-concentrated in DEPC-treated H_2O to get stock solutions with a concentration 100pg/µl. A working solution (10pg/µl) was prepared for each primer to carry on the PCR reaction.

Table 2.3 Sequences of primers used for cloning

gene	Sequences 5' to 3'	Restriction enzyme	Cloning vector
Human's genes			
CB_1	Forward: TAAAAGCTTGGCGCCCTAACCCTGGATTGCCCC Reverse: TAAGGATCCTGCGGACGGCGTGGCTGTGAGC	HindIII BamHI	pBluescriptII SK(-)
CB_2	Forward: TAAAAGCTTCCCGGCAGCTCCCAGTGCCCA Reverse: TAAGGATCCGGGCCTGGAGGCTCGCTTTGGC	HindIII BamHI	pBluescriptII SK(-)
Rat's genes			
Nurr1	Forward: TAAAAGCTTACTCCAATCACTCGGCTGAA Reverse: TAAGCGGCCGCTCAGCCAGCTCTCTACCCAT	NotI HindIII	pcDNA3.1 (+)
IL-10 promoter	Forward: TAACTCGAGAGAGGGCAGTGAGGGTCCAC Reverse: TAAGATATCTGCTGAGCCAGGCATGCTGAA	XhoI EcoRV	pGL4.10 [luc2]

2.11.2 Amplification of the synthesized cDNA

For cloning, fragments of the CB_1, CB_2, Nurr1 and IL-10 promoter were amplified using PCR (Bio-Rad, USA) along with the designed primers according to the procedure published in the Phusion DNA Polymerase kit (New England Bio, UK). The recipe for the reaction is given in Table 2.4.

Table 2.4 Recipe for the PCR.

Reagents	Amount (µl)
PCR buffer (10X)	10.0
10mM dNTPs	2.0
Forward primer (100pM)	1.5
Reverse primer (100pM)	1.5
DNA template	4.0
Phusion Taq polymerase	0.5
Nuclease free water	Up to 50

The following PCR conditions were used for the reaction

1. 98ºC for 2min

2. 98ºC for 30sec

3. 64ºC for 30sec

4. 72ºC for 2min

Repeat step from 2 to 4 for 30 times

5. 72ºC for 10min

Following the PCR reaction, an appropriate amount of loading dye was added to the samples and subsequently electrophoresed on a 1.0% agarose gel (Sigma, USA), stained with Sybr-green (Sigma, USA) in the presence of 1Kb molecular weight ladder (Invitrogen, #15628). The gel was observed under UV dark reader (UVP Bioimaging Systems).

2.11.3 Restriction endonuclease double digestion of PCR fragments and plasmid expression vector

Vectors and the PCR products were digested with appropriate restriction enzymes (see Table 2.3). All enzymes are ordered from NEB. The recipe for the digestion reaction is shown in Table 2.5.

Table 2.5 Recipe for the digestion reaction.

Reagents	**Amounts (µl)**	**Final amounts**
Restriction enzyme I	1.0	10 units
Restriction enzyme II	1.0	10 units
Buffer (10X)	5.0	1X
Vector or insert	1µg	
water	Up to 50	

The reaction was incubated at 37ºC for 2h. Then, 50µl was loaded onto a 1% agarose gel. The insert and vector were cut from the gel and extracted using QIAquick Gel Extraction Kit (Qiagen, #28704) according to protocol described below.

2.11.4 Extraction of DNA from agarose gel

The DNA extraction was performed as follows: gel fragment was solubilised with proper volume of Buffer QG and heated to 50ºC for 10min. The samples passed through the QIAquick column by centrifugation for 1min. After flow-through was removed, 500µl of Buffer QG were added to the column and centrifuged for 1min, then flow-through was discarded. To wash the column, 750µl of PE Buffer were added and centrifuged for 1min. To elute DNA, the columns were placed into a clean tube, and then 30µl of EB Buffer were added to column's membrane and centrifuged for 1min.

2.11.5 Ligation

Roche Rapid DNA Ligation Kit (Biolab, #02025) was used to generate recombinant vectors carrying CB_1, CB_2, Nurr1 variants and IL-10 promoter. 10 ng of appropriate vectors and 50ng of insert were used for each ligation reaction. Inserts and vectors were mixed with 1µl of DNA ligation buffer and 1µl (1U) of T4 DNA ligase, the volume is completed up to 10µl by nuclease free H_2O. The reaction was incubated overnight at 16˚C.

2.11.6 Transformation

50µl of TOP-10 *E.coli* competent cells (New England Biolabs, UK) were incubated with 2µl of ligation reaction on ice for 10min. The cells were then incubated at 42ºC

for 30sec and put on ice immediately for 5min. 950µl of SOC medium was added to the cells and incubated for 1h at 37ºC on a shaker at 250 rpm. The transformed cells were spread onto selective LB-agar plates containing 50µg/ml ampicillin and incubated for 24hrs at 37ºC. Then, 5 colonies from each plate were picked to check for the presence of the recombinant vector by colony PCR.

2.11.7 Colony PCR

The edge of the colony was gently touched with a 10µl pipette tip and re-suspended into the PCR reaction. DNA polymerase (BIOTAQ, #21040) was used for the amplification whose recipe is given in Table 2.6.

Table 2.6 Recipe for colony PCR.

Reagents	**Amount (µl)**
PCR buffer (10X)	2.0
10mM dNTPs	1.0
Forward primer	1.0
Reverse primer	1.0
Polymerase	0.5
Nuclease free water	Up to 20µl

PCR amplification steps were as following;

1. 98ºC for 1min
2. 98ºC for 20sec
3. 60ºC for 30sec
4. 72ºC for 50sec

Repeat step from 2 to 4 for 34 times

PCR reaction products were then loaded onto 1% agarose gel to check the insert amplification. Colonies which carried the corresponding recombinant vector were picked and inoculated in 5ml of LB growth medium and incubated at 37ºC on a shaker overnight. 4ml of bacterial culture was centrifuged to use for the extraction of the recombinant vector according to the mini-prep protocol (QIAprep Spin Miniprep Kit, QIAGEN, #27104) as the following: the bacterial pellet was re-suspended in 250µl buffer b1 by vortexing, then 250µl of buffer b2 and 350µl of buffer N3 were added, and the lysate was mixed by inversion. The lysate was spinned at 17,000xg for 10min at 4˚C and the supernatant was taken into a clean tube. 800µl of the supernatant was applied from the previous step to the spin column and centrifuged for 1min. 0.75ml Buffer PE was used to wash the spin column with and centrifuged for 1min. DNA was eluted in 50µl Buffer EB.

The recovered recombinant vectors were subjected to restriction enzyme double digestion using the appropriate restriction enzymes. The digestion product was then analysed for the identification of insert and vector using ethidium bromide 1% agarose gel in presence of 1kb ladder. The concentration of recombinant vectors was measured spectrophotometrically, and then a concentration of 100ng/ml was prepared and sent for sequencing. Cloning process is summarized in Figure 2.1.

2.11.8 Plasmid DNA purification using caesium chloride (CsCl)

CsCl Isolation of Plasmid DNA by gradient ultracentrifugation was carried out to purify closed circular plasmid DNA. *E. coli* cells stock was inoculated on LB agar and incubated overnight at 37˚C. A single colony from the LB agar was inoculated in 5ml LB broth and incubated for 6hrs at 37˚C. 100µl of LB culture were transferred to

100ml LB media and incubated overnight at 37˚C. To isolate the plasmid, the bacterial culture transferred into two 50ml tubes and centrifuged at 6000 rpm for 15min at 4˚C. The pellets were re-suspended in 5ml ice-cold alkaline solution I (50mM glucose, 25mM Tric-HCl, 10mM EDTA) by vortexing, then 5ml of freshly prepared alkaline lysis solution II (0.2M NaOH, 1.0% SDS) and 5ml of alkaline solution III (5M potassium acetate, 11.5% glacial acetic acid) were added, and then the lysate was mixed by inversion and incubated on ice for 5min. The lysate was centrifuged at 13,000rpm for 5min at 4˚C and the supernatant was transferred to clean tube. To precipitate the nucleic acids, a double volume of isopropanol was added and left at -20˚C for 1 hour, and then the mixture was centrifuged at 5000 rpm for 15 min at 4˚C, the pellet was then re-suspended in 5ml of 0.2M sodium acetate (pH 5.2). To precipitate the RNA, 5ml of ice-cold 5M LiCl were added and incubated on ice for 30min. To pellet the RNA, the solution was centrifuged at 5000rpm for 15min at 4˚C, and the supernatant was transferred to a new clean tube. DNA was precipitated by adding 2.5 the volume of 100% ethanol and left the solution for 1 hour at -20˚C. The DNA was collected by centrifugation at 10,000rpm for 15 min at 4˚C. The pellet was then washed with 70% ethanol and re-suspended in 4.5ml of 1.0g/ml freshly prepared and filtered CsCl solution containing 100µl of 10mg/ml ethidium bromide. DNA/CsCl solution was transferred to 5ml polyallomar tube, and then tops the solution with mineral oil layer to remove the air bubbles. The solution was subjected to ultracentrifugation at 54,000rpm for 14hrs at 22˚C. Gradient ultracentrifugation produced two bands; under UV light, the lower band that contained the desired closed circular plasmid DNA was transferred into new 1.5ml tube. To extract the ethidium bromide, the solution was washed sequentially with equal volumes of isoamyl alcohol 3 times, the bottom layer that contains the DNA plasmid was transferred to new 5ml

tubes and the volume adjusted to 2ml with H_2O. To precipitate the DNA plasmid, 200µl of 3M sodium acetate and 2ml of isopropanol were added, and then the solution was incubated for 1h at -20˚C. To recover the plasmid DNA, the solution was centrifuged at 10,000rpm for 15min at 4˚C, and then the pellet was washed two times with 70% ethanol; finally the pellet was re-suspended in 100µl DEPC treated H_2O. The concentration of plasmid was measured spectrophotometrically and the DNA plasmid was stored at -20˚C.

2.11.9 Nurr1 variants cloning

To amplify all known Nurr1 isoforms, a PCR reaction was performed on rat samples with Phusion Taq polymerase using a forward (within exon 2) and reverse primers (within exon 8) (see Table 2.3). The reaction was initiated by incubation at 95˚C for 2min, then 35 cycles of 95˚C for 30sec, 59˚C for 45sec, 72˚C for 2min, followed by a final cycle of 72˚C for 10min. 10 PCR reactions were carried out and combined into new tube, then 50µl 3M Sodium Acetate PH 5.2 and 1ml absolute ethanol were added and the solution was incubated for 1h at -20ºC for DNA precipitation. The sample was centrifuged at 17,000xg for 20min at 4ºC to precipitate DNA. The DNA pellet was washed with 500µl of 70% ethanol then centrifuged at 17,000xg for 20min at RT. The pellet was re-suspended in 20µl nuclease free H_2O, then separated on a 1.5% agarose gel, stained with Sybr green and imaged with UV dark reader (UVP Bioimaging Systems).

To create the Nurr1 isoforms expression constructs, the bands were cut from the gel and extracted using QIA-quick Gel Extraction Kit (Qiagen, #28704) according to protocols as described in section 1.11.4. The Nurr1 variants as described above were

ligated into pcDNA3.1(+) and transformed into *E. coli* competent cells. Plasmids with inserts from many colonies were analysed by sequencing to recognize the individual splice variants. Sequence comparison was carried out using the Blast from NCBI.

2.11.10 IL-10 promoter cloning

To clone the rat IL-10 promoter region (-1.6kb upstream the start codon). The PCR product amplified using the two primers described in Table 2.3. The promoter region was amplified via PCR from rat genomic DNA, and the PCR product was purified by QIAquick Gel Extraction Kit gel. The purified PCR product was ligated into the multiple cloning site of pGL4.10 luc2 upstream of the coding region for firefly luciferase. To check the correct orientation of the inserted promoter region, sequencing or restriction enzyme digestion were carried out. Sequence comparison was carried out using the Blast from NCBI.

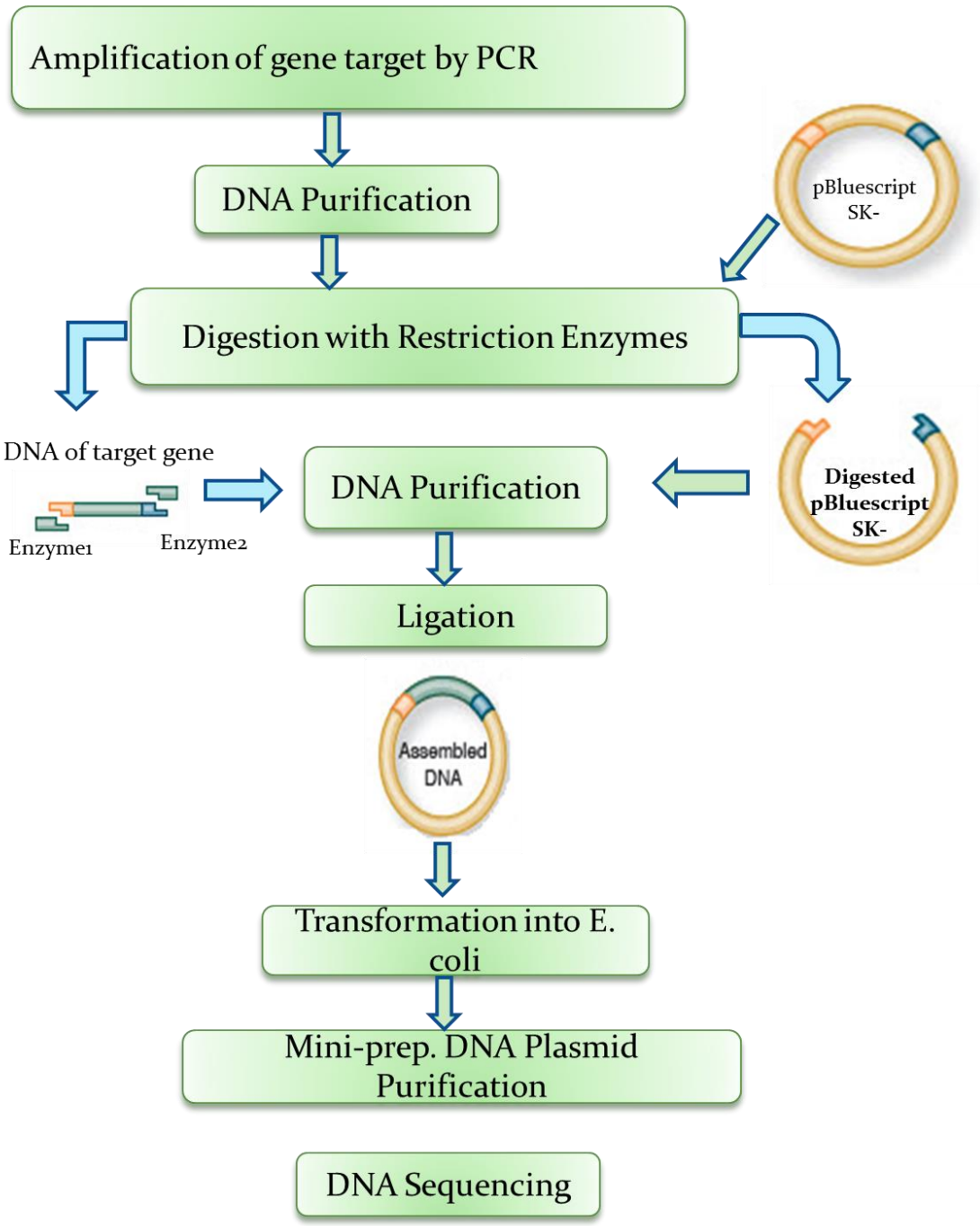


Figure 2.1 Flow chart of Cloning to insert target sequence into vector plasmid.

2.12 In situ hybridization

2.12.1 Labelling of the oligonucleotides

The aim of labelling is to prepare a digoxigenin (DIG) labelled probe that can be detected once bound to the target mRNA within the tissue, Figure 2.2 illustrates the DIG imaging system.

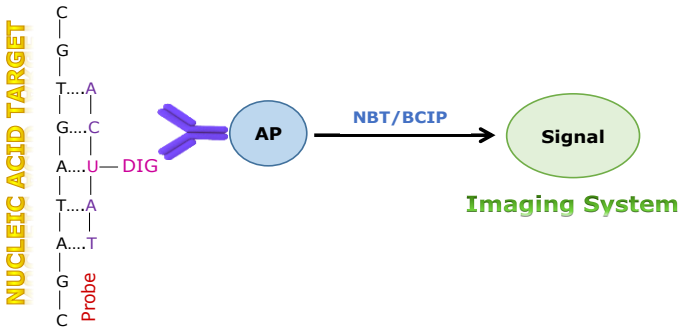


Figure 2.2 Schematic diagram showing the detection of DIG-labelled nucleic acids using chemiluminescence substrates. The target nucleic acids are hybridised to a DIG-labelled probe. Subsequent detections are carried out using anti-DIG antibodies conjugated to alkaline phosphatase.

Template DNA was prepared by linearizing 2.0µg of plasmid with HindIII restriction enzyme for 2 hours at 37˚C. The linear template was cleaned up by adding equal volume of phenol/chloroform/isoamyl alcohol and mixed by vortexing. The mixture was centrifuged at maximum speed for 3 min at RT and the upper aqueous layer then was transferred to a new tube. To precipitate the linearized plasmid, 1/10 of mixture volume from the sodium acetate 3M (PH 5.2) was added, 1µl of glycogen and two volumes of isopropanol, and then the mixture was incubated at -20˚C for 30min. DNA plasmid was pelleted by centrifugation at 17,000xg for 15min at 4˚C and the pellet was washed twice by 70% ethanol. The pellet was re-suspended in 20µl DEPC treated H_2O; 5µl were taken and run on 1.0% agarose gel to check that the plasmid is fully

digested. In vitro transcription reaction was carried out as follows: approximately 1µg of the linear plasmid was incubated at 37˚C for 2hrs with 2µl 10x transcription buffer, 2µl DIG labelled nucleotide mix, 2µl T3 RNA polymerase and 0.5 RNase inhibitor and the final volume was completed to 20µl with DEPC treated water. An aliquot (0.5µl) was removed and run on 1.0% agarose gel to check probe was produced (see figure 2.3). The final volume of the labelled probe was made to 100µl with 10mM DTT and stored at -80˚C in 20µl aliquots until further use.

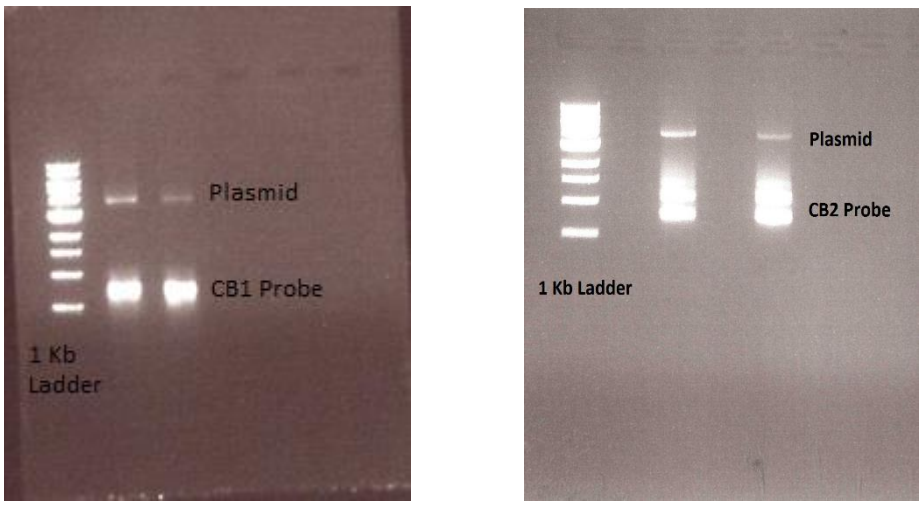


Figure 2.3. 1.0% agarose gel showing CB_1 and CB_2 probes ~600-800pb.

2.12.2 Brain tissue preparation

10µm thick sections were sliced from each tissue block and placed on slides. The slides were de-paraffinized with xylene 3 times for 5min each and hydrated with descending alcohol series (100%, 95%, 70% and 50%, 5 min each). The slides were then washed with DEPC treated H_2O twice for 8min each.

2.12.3 Preparation of hybridization buffer

One hundred and twenty eight slides were used to carry out in situ hybridization. Dextran sulphate was dissolved in a solution containing 10x salts (2M NaCl, 50mM EDTA, 100mM Tris-HCl, 50mM $NaH_2PO_4.2H_2O$, 50mM NaH_2PO_4), 50x Denhardt's (1.0% BSA, 1.0% polyvinylpyrrolidone, 1.0% Ficoll), 50% deionized formamide and 0.1mg/ml yeast tRNA. Once the dextran sulphate was completely dissolved, the solution was toped up with DEPC water to the desired volume.

2.12.4 Hybridization

DIG-labelled probes were diluted 1/1000 in pre-warmed (65˚C) hybridization buffer. Slides were completely covered with 400µl of hybridization buffer and diluted probe, and then the slides were cover slipped and hybridized overnight at 65˚C in a humidified chamber. As a negative control, one slide was covered only with hybridization buffer; furthermore luciferase probe was used as sensitive probe.

After incubation the coverslip was gently removed and the slides were washed twice in MABT buffer (100mM maleic acid, 150mM NaCl and 0.1% tween-20 PH 7.5), followed by 2 washes with wash buffer (1x saline-sodium citrate (ssc), 50% formamide and 0.1% tween-20) at 65˚C, each wash 30min. To remove traces of the formamide, slides were then washed twice in MABT, each wash 5min. The slides were then kept in a humidified chamber with blocking solution (2% blocking reagent (Roche, Cat#1096176), 10% heat inactivated sheep serum and MABT) for 1 hour at RT. To develop the in situ signal, the slides were incubated overnight at 4˚C with alkaline phosphatase (AP) conjugated anti-DIG (Roche, Cat#1093274) diluted 1/1500 in antibody buffer (100mM Tris-HCl, 150mM NaCl, 1% blocking reagent and 1% heat

inactivated sheep serum). After antibody incubation, the slides were washed 3 times in MABT, each wash 15min. The colour was developed by incubating the slides in pre-developing buffer (100mM Tris (PH 9.8), 100mM NaCl and 50mM $MgCl_2$) twice, each wash lasted 2min. Pre-developing buffer was replaced with developing buffer (100mM Tris (PH 9.8), 100mM NaCl, 50mM $MgCl_2$, 5% polyvinyl alcohol, 0.11mM 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Roche, Cat#1383213) and 0.12mM nitroblue tetrazolium salt (NBT) (Roche, Cat#1585002)), slides were incubated in developing buffer overnight at 37˚C, until the blue colour was produced. Subsequently, the slides were dehydrated in an ascending ethanol series, 30sec in each of 50%, 70%, 90% and 100% ethanol respectively, followed by 2 washes with xylene, and then they mounted in Depex. The slides were examined under a bright field microscope (Leica DM4000B). Chromogenic in situ hybridization protocol is summarized in Figure 2.4.

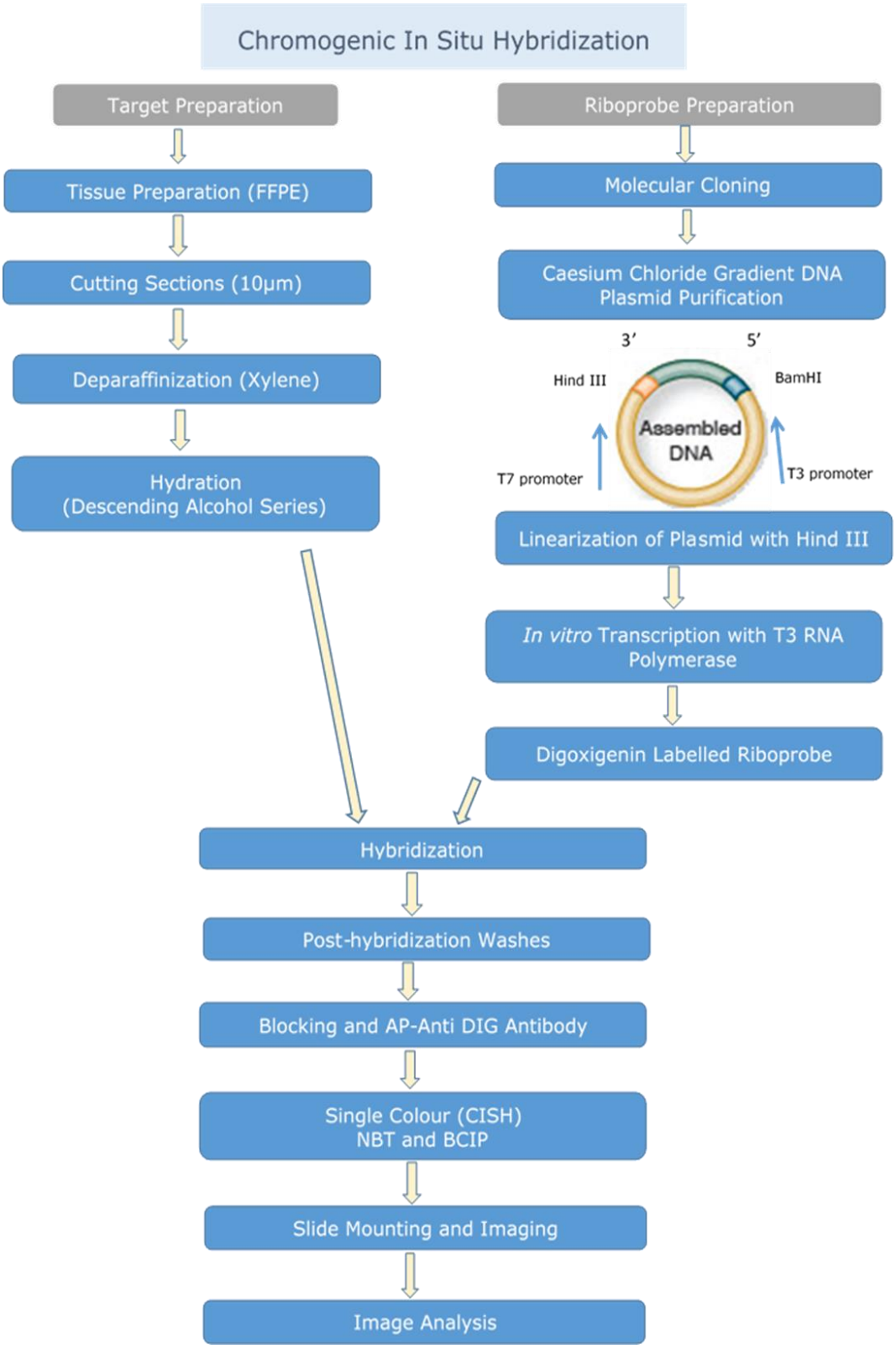


Figure 2.4 Scheme summarizing the in situ hybridisation process of CB_1 and CB_2 receptors.

2.13 Gene Reporter Vectors

pGL4.10 luc2 vector was used as reporter vectors. IL-10 and iNOS promoters were cloned into pGL4.10 luc2 vector, IL-10 promoter was cloned in house, while iNOS promoter was a kind gift from Dr Dawson (University of Nottingham).

2.13.1 Transfection

The mouse macrophage cell line (Raw 246.7) was transfected using X-tremeGENE HP DNA transfection reagent (Roche, #25174600) with recombinant reporter vectors. The transfection was performed in 12-well plate format. The day before transfection 1.25 x 10^5 cells were plated per well in a 12-well plate in 1ml DMEM (D5671, Sigma) containing 10%FBS and 1% antibiotics mix (penicillin/streptomycin) and incubated for 24h at 37˚C in 5% CO_2 incubator. Cells were then transfected using 3µl transfection reagent per 1µg DNA (ratio 1:3, DNA: X-tremeGENE) after leaving the transfection complex for 30min at RT. Enhanced green fluorescent protein vector (eGFP) was employed as a reporter gene for successful plasmid DNA transfection, the transfection efficiency was 30% throughout the study. After 16hrs of incubation at 37˚C, LPS, ATP or HMGB1 were added to relevant wells and the media was changed for the rest and then incubated again for 24hrs at 37˚C in 5%CO_2 incubator.

2.13.2 Luciferase reporter assay

A luciferase assay (Promega, #E1941) was used to measure the luciferase activity within the transfected cells. 48hrs after transfection, media was aspirated and cells were washed once with 1ml of PBS. Then, cells were incubated in 250µl of 1X Passive Cell Lysis Buffer on orbital shaker for 45min at RT for cell lysis. 20µl of the lysate were transferred to luminometer tubes and mixed with 100µl of Luciferase Assay

Reagent, then vortexed for 10sec and luciferase activity was measured for 10sec using the luminometer (TD20/20, Turner Designs).

2.14 Gene knockdown assay

The siRNA reagents were purchased from Dharmacon (Cat # E-091528-00-0010). Knockdown of Nurr1 was carried out according to the manufacturer's instructions. Briefly, microglial cells were plated at a density 2.0 x 10^5 cells/ml of medium onto 12-well plates and incubated at 37˚C in CO_2 incubator, and were used for experiments within 24hrs after plating. Nurr1 knockdown was validated on the protein and mRNA levels.

2.15 Lentivirus production

2.15.1 Create entry clones

2.15.1.1 Designing attB PCR primers

To produce PCR products suitable for use as substrates in a Gateway BP recombination reaction with a donor vector, attB sites were incorporated into the PCR products. To enable efficient Gateway cloning, the forward and reverse primers were required to contain four guanine (G) residues at the 5′end, followed by 25bp attB1 site, followed by 18–25bp of gene-specific sequences, see Table 2.7.

Table 2.7 Sequences of primers used to attB PCR products.

Nurr1 variants	**Sequences 5' to 3'**
Nurr1	Forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCATGCCTTGTGTTCAGGCG Reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAGAAAGGTAAGGTGTCCAGG
TINUR	Forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCATGGACAACTACAGCACAG Reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAGAAAGGTAAGGTGTCCAG
Nurr1a	Forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCATGCCTTGTGTTCAGGCG Reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTTTCATATTCTGTATGCTAAGCGTAGAAC
GFP	Forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCATGGTGAGCAAGGGCGAGGA Reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTTTTACTTGTACAGCTCGTCCATGCCGA

2.15.1.2 Producing attB-PCR products

Phusion HF Polymerase (New England Bio, UK) was used for PCR to prepare attB-PCR Products. The recipe for the reaction is given in Table 2.8.

Table 2.8 Recipe for attB-PCR reaction.

Reagents	**Amount (µl)**
5X Phusion HF buffer	10.0
10mM dNTPs	1.0
Forward primer (10mM)	2.5
Reverse primer (10mM)	2.5
Template	4.0
Enzyme	0.5
Nuclease free water	29.5

The following PCR conditions were used for the reaction

1. 98ºC for 30sec

2. 98ºC for 30sec

3. 64ºC for 30sec

4. 72ºC for 1min

Repeat step from 2 to 4 for 34 times

5. 72ºC for 5min

Electrophoresis was carried out in 1% agarose gel made in 1x TAE buffer with ethidium bromide. PCR reactions were loaded directly on the gel and 1kp DNA ladder (Invitrogen, #15628) was also loaded onto the gel as a marker. DNA was visualised using a UV transilluminator (UVP Bioimaging Systems).

2.15.1.3 Purifying attB-PCR products

100µL of 30% Polyethylene Glycol (PEG) 8000/3mM $MgCl_2$ and 150µL of TE (pH 8.0) were added to a 50µL amplification reaction containing attB-PCR product. The mixture was mixed thoroughly and centrifuged at 10,000×g for 15min at RT. The supernatant was aspirated and the pellet was dissolved in 50µL of TE, pH 8.0 (to concentration > 10ng/µL). The quantity and quality of the attB-PCR product was checked on a 1% agarose gel.

2.15.1.4 Entry clones creation using the BP recombination

2µL of BP Clonase II enzyme mix was added to the sample and positive control (pUC19) vials and mixed well by vortexing twice for 2sec each time, and then the

reactions incubated overnight at 25°C. To end the reaction, 1µL of the Proteinase K solution was added to each reaction, and then incubated for 10min at 37°C.

2.15.1.5 Transforming the entry clone to competent *E. coli*

One vial of TOP-10 (New England Biolabs, UK) was thawed on ice for each transformation. 1µL of the BP recombination reaction was added into a vial of ccdB cells and mixed gently. For the pUC19 positive control, 10pg of DNA were added into a separate vial and mixed gently. The vials were placed on ice for 30min, then the cells Heat-shocked for 30sec at 42°C. The cells were removed from the 42°C bath and incubated on ice for 2min. 250µL of S.O.C. medium were added to each vial. The vials were shake horizontally (225rpm) at 30°C for 1h. Before plating, the transformation mixture was diluted 1:10 into LB Medium. 50µL were spread from each vial on a pre-warmed selective plate and incubated overnight at 30°C.

Colony PCR was carried out to detect the colonies that carry the recombinant reaction using M13 sequencing primers (M13 Forward: GTAAAACGACGGCCAG, M13 Reverse: CAGGAAACAGCTATGAC). Colonies which carries the corresponding entry clone were chosen and inoculated in 5ml of LB medium containing 50µg/ml kanamycin and incubated at 30ºC on a shaker for overnight. 500µl of bacterial culture were mixed with same volume of 50% glycerol and stored at -80ºC. 4ml of bacterial culture were used for the extraction of the entry clone according to the mini-prep protocol as described above, and sent for sequencing.

2.15.2 Expression clone creation using the LR recombination reaction

To create an expression clone, LR recombination reaction was performed using the proper attL and attR-containing substrates, then the reaction mixture was transformed into an appropriate *E. coli* host and selected for entry clones via sequencing PCR.

To carry out a LR recombination reaction, the entry clones and the pENTR™-gus positive control linearise by restriction enzyme digestion EcoRV. The Gateway LR Clonase II enzyme mix was removed from −20°C and thawed on ice for 2min and vortexed briefly twice for 2sec each time. 2µL of Clonase II enzyme mix were added to the sample and positive control vials and mixed well by vortexing twice for 2sec each time. The reactions were incubated at 25°C for overnight. To terminate the reaction, 1µL of the Proteinase K solution was added to each reaction and incubated for 10min at 37°C.

Equal amounts (50pg) were transformed of destination vector into competent Stbl3 cells (Invitrogen, C737303) using the protocol that mentioned above. Also 50pg of the positive control plasmid were transformed. The bacterial culture was diluted 1:10 and Plated onto selective plates containing ampicillin and incubated overnight at 30ºC. The pUC19 positive control DNA was used to verify that the transformation efficiency. Colony PCR was carried out to detect the colonies that carry the destination vector the following primers; LNCX Forward: AGCTCGTTTAGTG-AACCGTCAGATC, PIND20 Reverse: GGTTACTCCA- GACTGCCTTGG. Colonies which carry the corresponding destination vector were selected and inoculated in 5ml of LB medium containing 50µg/ml ampicillin and incubated at 30ºC on a shaker for overnight. 500µl of bacterial culture were mixed with 500µl of 50%

glycerol and stored at -80ºC. 4ml of bacterial culture were used for the extraction of the entry clone according to the mini-prep protocol as described above, and sent for sequencing.

2.15.3 Production and purification of lentiviral vectors

Packaged plasmids were transfected into HEK293FT (human embryonic kidney cell line), lentiviral particles accumulated in the supernatant and high-titer viral preparations were achieved by ultracentrifugation. The details viral production protocol as follows:

Day 1: HEK293FT cells seeding

1.2 x10^6 HEK293FT cells were plated in 10cm dishes using 10ml of DMEM media (Sigma, D5671) with 10%FBS, to get ~50% confluency. The cells were incubated at 37˚C for 24hrs.

Day 2: Transfection with plasmid mix

To a 2mL centrifuge tube, 600µl OPTIMEM media were placed, then 2µg from each plasmid DNA (Nurr1 isoforms expression vector or GFP positive control vector, packaging plasmid (PMD2G) and envelope plasmid (PSPAX) were added, 18µl of X-tremeGENE HP transfection reagent were added to the diluted DNA (3:1 ratio of reagent to DNA) and then the transfection complex was mixed gently by pipetting and incubated for 30min at RT. The cell media was removed and replaced by 7ml OPTIMEM, the cells were transfected with 600µl of transfection complex drop by drop around the dish. The dishes were mixed gently and incubated for 6hrs at 37˚C in 5%CO_2 incubator. The culture media was aspirated after 6hrs, 10ml fresh DMEM

media with 10% FBS were added and the cells incubated for 48hrs at 37˚C in 5%CO_2 incubator.

Day 4: Lentivirus collection

The supernatant (10ml) that containing the lentivirus was collected and filtered through a 0.45µm filter. The filtered supernatant was stored at 4˚C up to 2 weeks.

Day 5: Concentrate the viral preparation

The supernatant was centrifuged at 100,000xg for 2hrs at 4°C. Without disturbing the pellet the supernatant was poured out and allowed the residual liquid to drain by standing the inverted tubes on absorbent paper towels and remaining droplets were aspirated. The viral pellets were re-suspended in 300µl of 1× PBS. The supernatant was transferred to a new tube and 20-µl aliquots of the supernatant were made and stored at –80°C.

In order to ensure that lentiviral medium is viable, and to assess the number of copies of viral constructs, the viral titer was carried out. Lentiviral supernatant was diluted in fresh culture medium to make the final volume 1ml for each sample, including culture medium as a negative control. 10µl of lentivirus reagent A were added and mixed by inverting. Then, 10µL of lentivirus reagent B was added and mixed. The mixture was incubated 30min at 37ºC, then centrifuged 5min at 12,000rpm. The supernatant was removed and the pellet was dissolved in 250µl of sample diluent. To inactivate the viruses, the mixture was vortexed and incubated 30min at 37ºC. The standard curve was prepared from a series of dilution of the recombinant HIV-1 p24 antigen in the concentration range of 100ng/ml – 1ng/ml by diluting the p24 stock solution in sample

diluent. A plate coated with anti-p24 antibody was incubated with 100µL of inactivated either lentiviral sample or standard p24 antigen at 4ºC overnight. The wells were emptied, and then the well strips were washed 3 times with wash buffer and dried. 100µl was added of the diluted FITC-conjugated anti-p24 monoclonal antibody to each well. The plate was covered and incubated at RT for 1h on an orbital shaker. Then, the plate cover was removed and the wells were emptied. Strip wells were washed 3 times as described above. 100µL of the diluted HRP-conjugated anti-FITC antibody were added to all wells, and the plate was covered and incubated at RT for 1h on an orbital shaker. The plate cover was removed and the wells emptied. The well strips were washed 3 times as described above. The substrate solution was warmed to RT, then 100µL of substrate solution were added to each well, including the blank wells. The plate was incubated at RT on shaker for 30min. 100µL of stop solution into each well were added in order to stop the enzyme reaction. Absorbance of each well was read on a spectrophotometer using 450nm.

2.15.4 Lentivirus infection

The neonatal and adult microglia were plated at a density 2.0 x 10^5 cells/ml of medium onto 12-well plates and incubated at 37˚C in CO_2 incubator, and were used for experiments within 24hrs after plating. The cells were infected with lentivirus for 4hrs, the medium was removed and added microglia culture media with doxycycline (1µg/ml) to induce the vector transcription. 48hrs post induction, the cells were treated with LPS for 6hrs, ATP and HMGB1 for 12hrs, the cells were harvested and RNA and protein were extracted to carry out the qPCR and Western blotting.

2.16 Statistical analysis

All statistics were calculated using GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA). Data were obtained from this study passed normality test for all the groups, so we compared differences using parametric tests. Data were obtained from three independent experiments with three triplicates from each experiments, and are represented as mean ± S.E.M. Differences were compared using the Student t test (two groups) or one-way analysis of variance (multiple groups) followed by post-hoc Bonferroni's multiple comparison test. The criterion for statistical significance was $P < 0.05$.

3 Chapter Three: Expression of the endocannabinoid system within human mid-brain and cerebellum tissues during postnatal development

3.1 Introduction

As discussed in Chapter 1, the endocannabinoid system is composed by cannabinoid receptors, endogenous ligands and biosynthesis and degradation enzymes (Salzet, 2000). Endocannabinoids are retrograde intercellular lipid messengers in GABAergic and glutamatergic neurons that are suggested to be produced by, and released from, postsynaptic neurones before travelling in a retrograde fashion across the synaptic cleft to act on cannabinoid receptors on the presynaptic neurones, thereby inhibiting neurotransmitter release (Wilson and Nicoll, 2002). The administration of endocannabinoids to laboratory animals causes distinct behavioural actions. For example, administration of anandamide produces effects including anti-nociception and catalepsy (Crawley et al., 1993, Calignano et al., 1998). The effects of endocannabinoids occur with a rapid onset, but with a short duration of action that is likely due to their rapid uptake into neurons and glial cells as well as enzymatic breakdown (Fride and Mechoulam, 1993, Smith et al., 1994).

3.1.1 The synthesis and degradation of endocannabinoids

Mammalian tissues express two endogenous endocannabinoids known as 2-AG and AEA which serve as endogenous ligands for the CB_1 and CB_2 (Pertwee et al., 2010). Unlike classical neurotransmitters, the endocannabinoids are not stored in vesicles within the cell prior to release but are synthesized on demand following cellular depolarization or receptor stimulation in a calcium-dependent manner (Di Marzo et al., 2004).

2-AG and AEA are both derivatives of arachidonic acid conjugated with glycerol or ethanolamine, respectively. Both are found in peripheral tissues and the CNS. The

concentration of AEA in the brain is 200-fold less than that of 2-AG, which indicates that although they have a common ability to activate endocannabinoid receptors; the physiological effects of these molecules may be different. (Stella et al., 1997, Porter et al., 2002). The synthesis of AEA involves enzymatic cleavage of the membrane phospholipid NAPE by the enzyme phospholipase D (PLD) that is found in different regions of brain including cerebrum and cerebellum (Okamoto et al., 2004). The synthesis of 2-AG involves production of 1,2-diacylglycerol (DAGL) and phosphatidylinositol (4,5)-biphosphate (PIP_2) via phospholipase C (PLC) (Stella et al., 1997). DAGL is placed in the plasma membrane and its distribution correlates with the abundance of 2-AG in tissues and organs (Carrier et al., 2004, Alexander and Kendall, 2007).

AEA and 2-AG act mainly via CB_1 and CB_2, which are both coupled to G_i/G_o proteins, which inhibit adenylyl cyclase with a subsequent reduction in intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels (Piomelli, 2003). They also couple positively to mitogen-activated protein kinases via these $G_{i/o}$ proteins, which inhibit voltage-activated Ca^{2+} channels and activate inwardly rectifying K^+ channels, these actions are related to the role of endocannabinoids as modulators of synaptic plasticity (Rubovitch et al., 2002, FAN and YAZULLA, 2003).

Endocannabinoid signalling is stopped by a two-step process, including transport into cells and hydrolysis by enzymatic systems. Both processes exhibit a control of endocannabinoid levels in tissues by rapidly removing these endocannabinoid molecules (De Fonseca et al., 2005). Endocannabinoid uptake into cells is facilitated by a transporter, in a process driven by concentration gradient across the membrane

(Beltramo et al., 1997). This transporter carrier is widely distributed throughout the brain, but it is not fully characterised (Giuffrida et al., 2001). Once the endocannabinoids enter the cell, they are exposed to rapid degradation mainly by two enzymatic systems: AEA by FAAH and 2-AG by MAGL (Di Marzo and Petrosino, 2007). FAAH belongs to the serine-hydrolase family of enzymes and is widely distributed through the CNS, especially in the cerebral cortex, hippocampus, cerebellar cortex, basal ganglia, red nucleus, the spinal cord (Tsou et al., 1998b). MAGL is also widely distributed in the nerve terminals of brain neurons including the axon terminals of granule cells, CA3 pyramidal cells and some interneurons (Gulyas et al., 2004). A summary of the biosynthesis, release and degradation of AEA and 2-AG is illustrated in Figure 1.3.

3.1.2 The expression of receptors, ligands and related enzymes of the endocannabinoid system within the CNS

Endocannabinoids are involved in many pathophysiological conditions, including pain, epilepsy, obesity and movement disorders (Fernández-Ruiz et al., 2000). The CB_1 receptor is expressed within the descending pain modulation circuitry, previous studies revealed that the CB_1 receptors localised in the PAG, RVM and spinal DH (Tsou et al., 1998a), and there is some evidence for non-neuronal CB_1 in B cells of the immune system (Kaplan, 2013), whereas CB_2 receptors are mainly found in peripheral tissue mainly in immune cells (Howlett et al., 2002). Some reports indicated the presence of CB_2 receptors in microglia and astrocyte of the CNS (Alkaitis et al., 2010), with some evidence suggesting neuronal CB_2 expression (Atwood and Mackie, 2010). However, GPR55 receptors are predominantly expressed in microglia (Pietr et al., 2009).

DAGLα is the most abundant endocannabinoid enzyme in the brain. Suarez and colleagues used in situ hybridisation and immunohistochemistry to reveal that DAGLα mRNA and protein are distributed and expressed in rat forebrain, hippocampus and hypothalamus (Suárez et al., 2011). On the other hand, NAPE-PLD has a wide distribution in various areas of rat brain such as brain stem, cerebellum and hippocampus, and its expression increase level is age-dependent (Morishita et al., 2005). The hydrolytic enzyme FAAH is present post-synaptically in neurons including the pyramidal cells of the cerebral cortex and hippocampus, and the Purkinje cells of the cerebellar cortex (De Fonseca et al., 2005).

3.1.3 Postnatal development of the endocannabinoid system

Several studies have revealed that the endocannabinoid signalling system plays a critical role during development and maturation (De Fonseca et al., 2005). In a recent study, Kwok et al. (2017) revealed that the endocannabinoids undergo postnatal change in rat brainstem nuclei, with the study assessing the expression of endocannabinoids synthesizing enzymes over postnatal age and finding that NAPE-PLD mRNA levels increased as the animals aged in PAG, whereas no changes were observed in DAGLα mRNA transcript levels. In addition, the authors reported that the expression of cannabinoid receptors also undergoes postnatal alteration, CB_1 mRNA transcript levels decreased not significantly as the animals aged in PAG and no changes in CB_1 mRNA levels were observed between ages in the RVM, however, the data obtained from this study revealed that no age-related changes were observed in CB_2 mRNA expression levels in both PAG and RVM. Taken together, these findings suggested that endocannabinoid signalling system has a crucial physiological function during postnatal development and the age-related alterations in the expression of

endocannabinoids throughout the postnatal period may be important for mediating the endogenous pain inhibition and maturation of synaptic connections through the pain signalling circuits. Moreover, these findings that indicated that the expression of endocannabinoids system undergoes postnatal refinements in rat raise questions whether these results are translational in humans.

3.1.4 Aims

It is critical to define the normal development of endocannabinoid signalling systems in human brain, and to clarify the changes in their various elements during critical periods such as embryonic and juvenile stages. Several studies have revealed that early postnatal pain perception is exaggerated and inappropriate (Andrews and Fitzgerald, 1994), which reflects the immaturity of pain signalling during the early development period of life (Fitzgerald and Jennings, 1999). The endogenous cannabinoid signalling pathways undergo noteworthy postnatal alteration (Belue et al., 1995, Kivell et al., 2004, Hathway et al., 2012, Long et al., 2012). The hypothesis of this chapter states that postnatal changes occur in endogenous endocannabinoid systems within human mid-brain and cerebellum during postnatal development, and that these changes are important in the maturation of pain processing during this period. Employing Taqman qPCR and in situ hybridisation techniques, this study aims to examine the developmentally regulated alterations in the endocannabinoid system in human mid-brain and cerebellum tissues.

3.2 Results

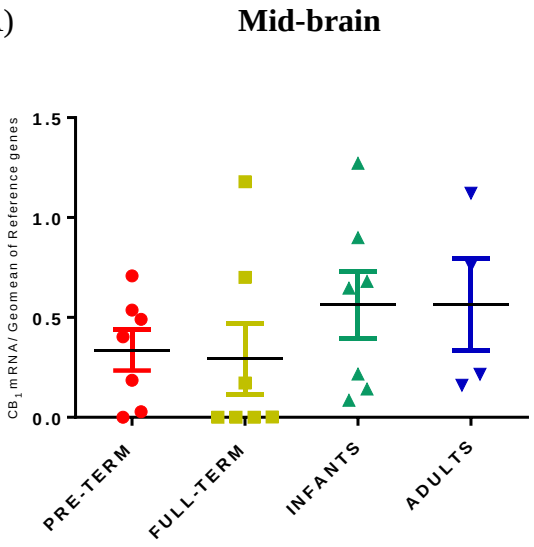
3.2.1 Changes in CB_1 receptor expression during postnatal development of the supraspinal centres

The expression of CB_1 within the mid-brain and cerebellum was investigated in pre-term, full-term, infant and adult human brain tissues using Taqman RT-PCR (n=8 for pre-term, full-term and infant, whereas n=4 for adult) and chromogenic in situ hybridization (n=4 for pre-term, full-term, infant and adult). No significant changes in CB_1 mRNA transcript levels was observed in mid-brain ($F(3,21)=0.7649$, $P=0.6094$; one-way ANOVA) (Figure 3.1a). CB_1 receptor mRNA transcript level in cerebellum was significantly higher at infant time points ($F(3,21)=8.187$, $P<0.0001$; one-way ANOVA; Bonferroni post-tests: infant vs. pre-term and infant vs. adult both $P<0.01$, whereas infant vs. full-term $P<0.001$; Figure 3.1b).

Similar to previously reported findings (Long et al., 2012), who described the development of CB_1 and some of the major endocannabinoid metabolic enzymes mRNA in human dorsolateral prefrontal cortex across postnatal life, cell counting of CB_1 positive cells in the midbrain indicated that the expression of CB_1 receptors is lowest in adults compared to earlier ages ($F(3,12)=8.945$, $P<0.01$; one-way ANOVA; Figure 3.2). Moreover, there were no differences in CB_1 receptors expression between pre-term, full-term and infant (Figure 3.2 and 3.3).

In situ hybridization staining demonstrated that the expression of CB_1 in cerebellum was higher in infants than in both preterm and full term (Figure 3.4 and 3.5). However, the CB1 expression in adult brain decreased but not significantly ($F(3,10)=3.684$, $P>0.05$; one-way ANOVA; Figure 3.4). In addition, in situ hybridization staining revealed that the number of CB_1 receptor positive cells in mid-brain was higher in all age tested compared with cerebellum.

A)



B)

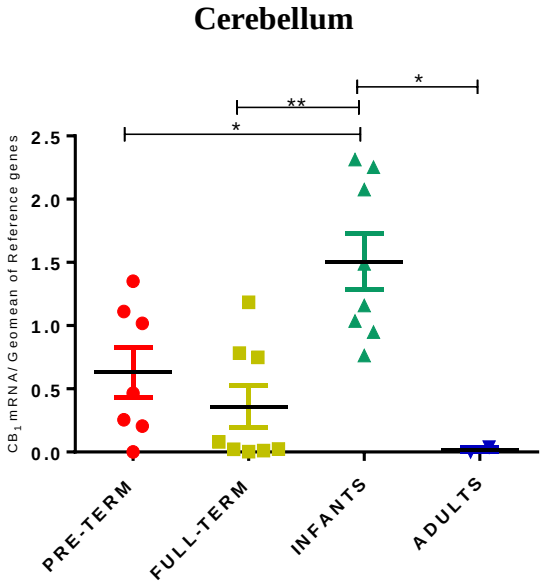
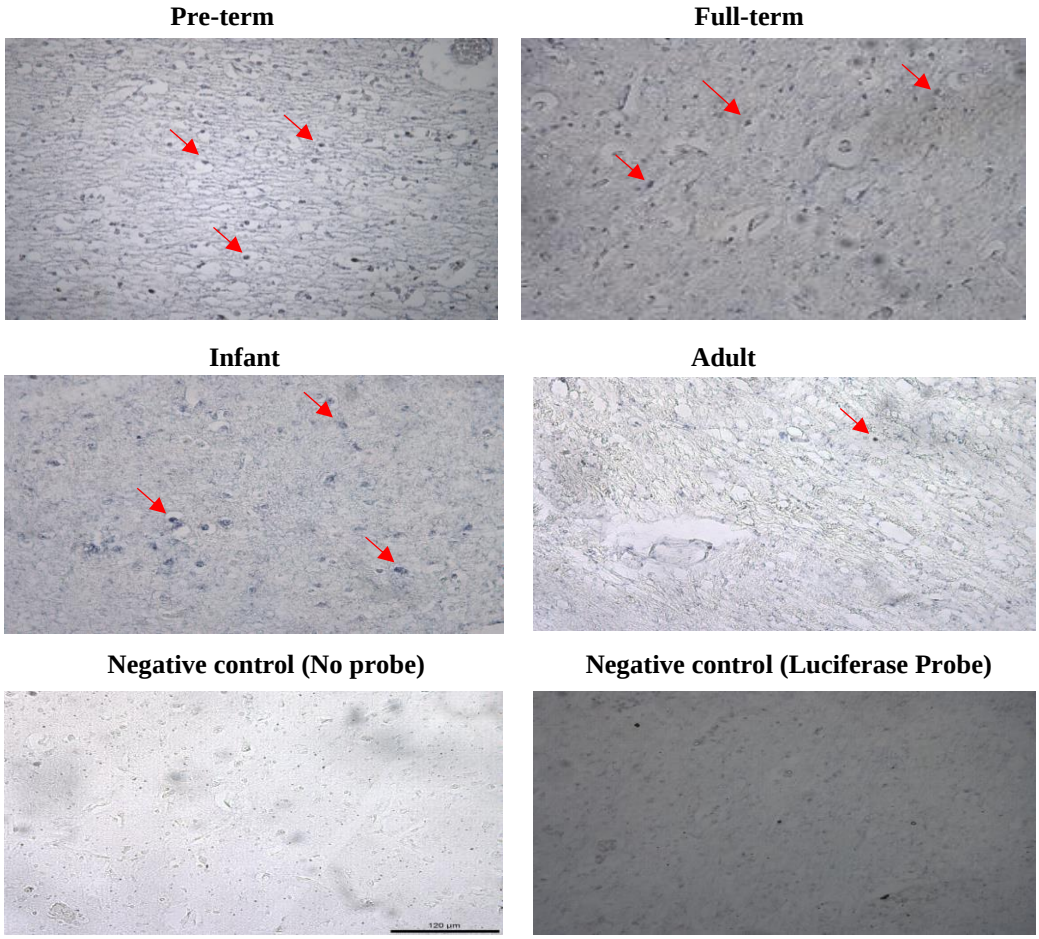


Figure 3.1 Taqman qPCR analysis of the expression of CB_1 receptor mRNA levels in (A) mid-brain and (B) cerebellum of pre-term, full-term, infant and adult human. The mRNA expression of CB_1 receptor in mid-brain did not change significantly over postnatal period. Whereas CB_1 receptor mRNA transcript level in cerebellum was peaked significantly at infant. Data represent mean ±SEM. *, ** = $P<0.05$ and $P<0.01$ respectively, one-way ANOVA with Bonferroni post-tests.

A)



B)

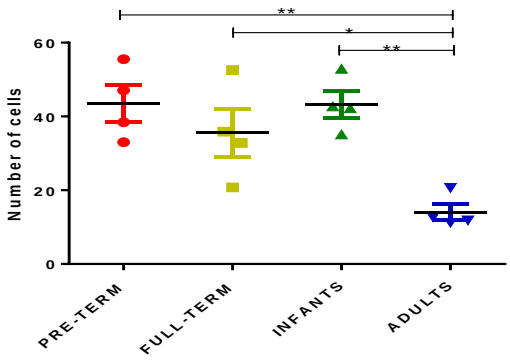


Figure 3.2. Chromogenic in situ hybridization localisation of CB_1 receptors in the <u>mid-brain</u> of pre-term, full-term, infant and adult human. (A) Upper panel shows in situ hybridization images (20X magnification), which were obtained using a bright field microscope with a camera attachment. (B) Lower panel shows quantification of in situ hybridization staining, cell counting were used where appropriate. The expression of CB_1 receptor in mid-brain did not change as the animals aged until the infant stage, whilst the expression of CB1 dropped down in adult brain. Data represent mean ± SEM. *, ** = $P<0.05$ and $P<0.01$ respectively, one-way ANOVA with Bonferroni post-tests.

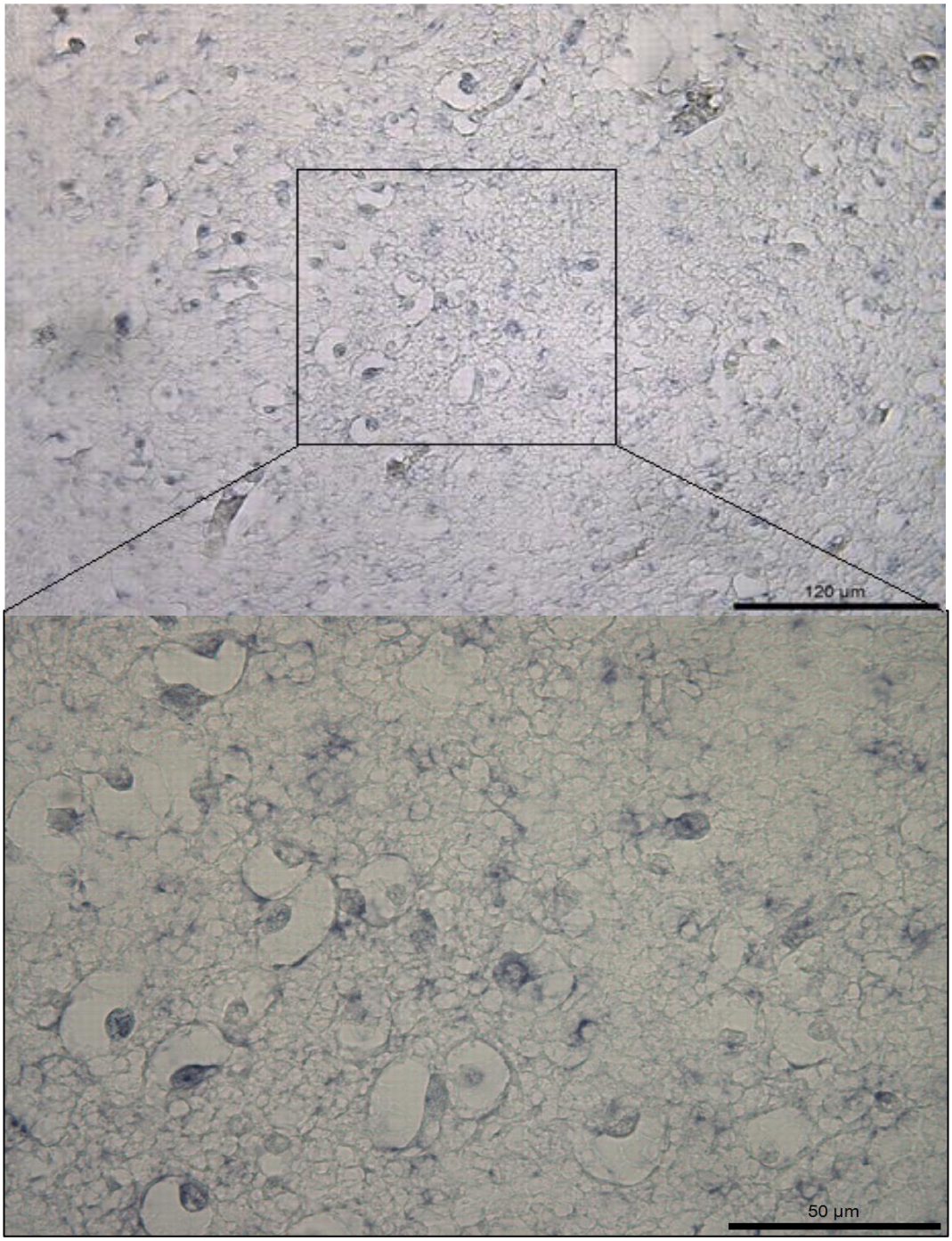
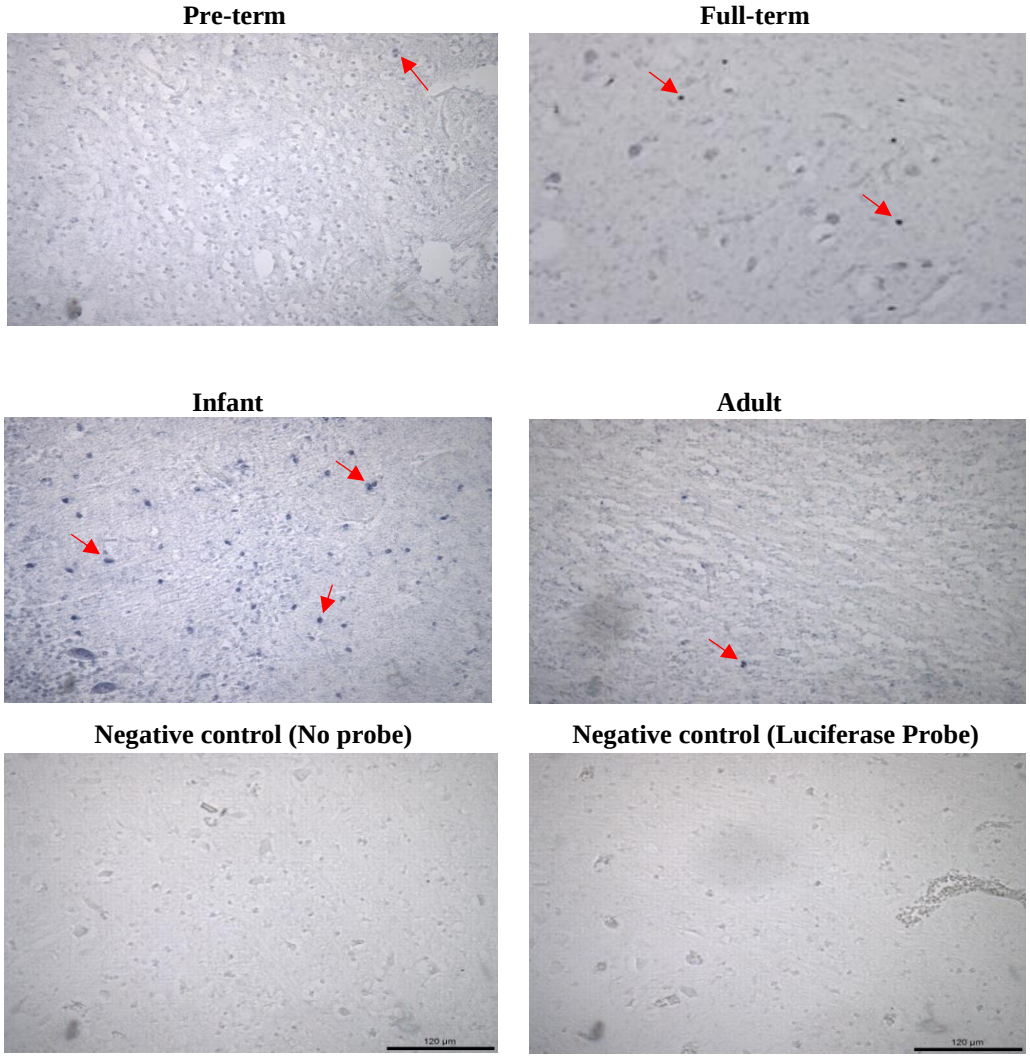


Figure 3.3. Chromogenic in situ hybridization localisation of CB_1 receptors in the <u>mid-brain</u> human. The figure shows in situ hybridization images with high magnification (50X), which were obtained using a bright field microscope with a camera attachment.

A)



B)

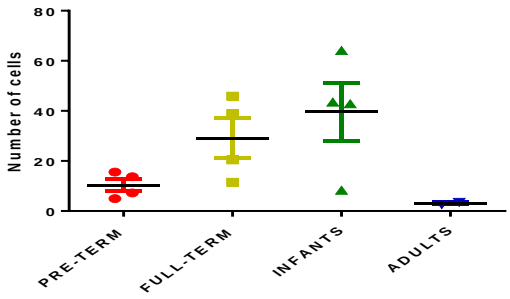


Figure 3.4. Chromogenic in situ hybridization localisation of CB_1 receptors in the <u>cerebellum</u> of pre-term, full-term, infant and adult human. (A) Upper panel shows in situ hybridization images (20X magnification), which were obtained using a bright field microscope with a camera attachment. (B) Lower panel shows quantification of in situ hybridization staining, cell counting were used where appropriate. The expression of CB_1 receptor in cerebellum increased as the animals aged until the infant stage, whilst the expression of CB1 dropped down in adult brain. Data shown here represent mean ± SEM, one-way ANOVA.

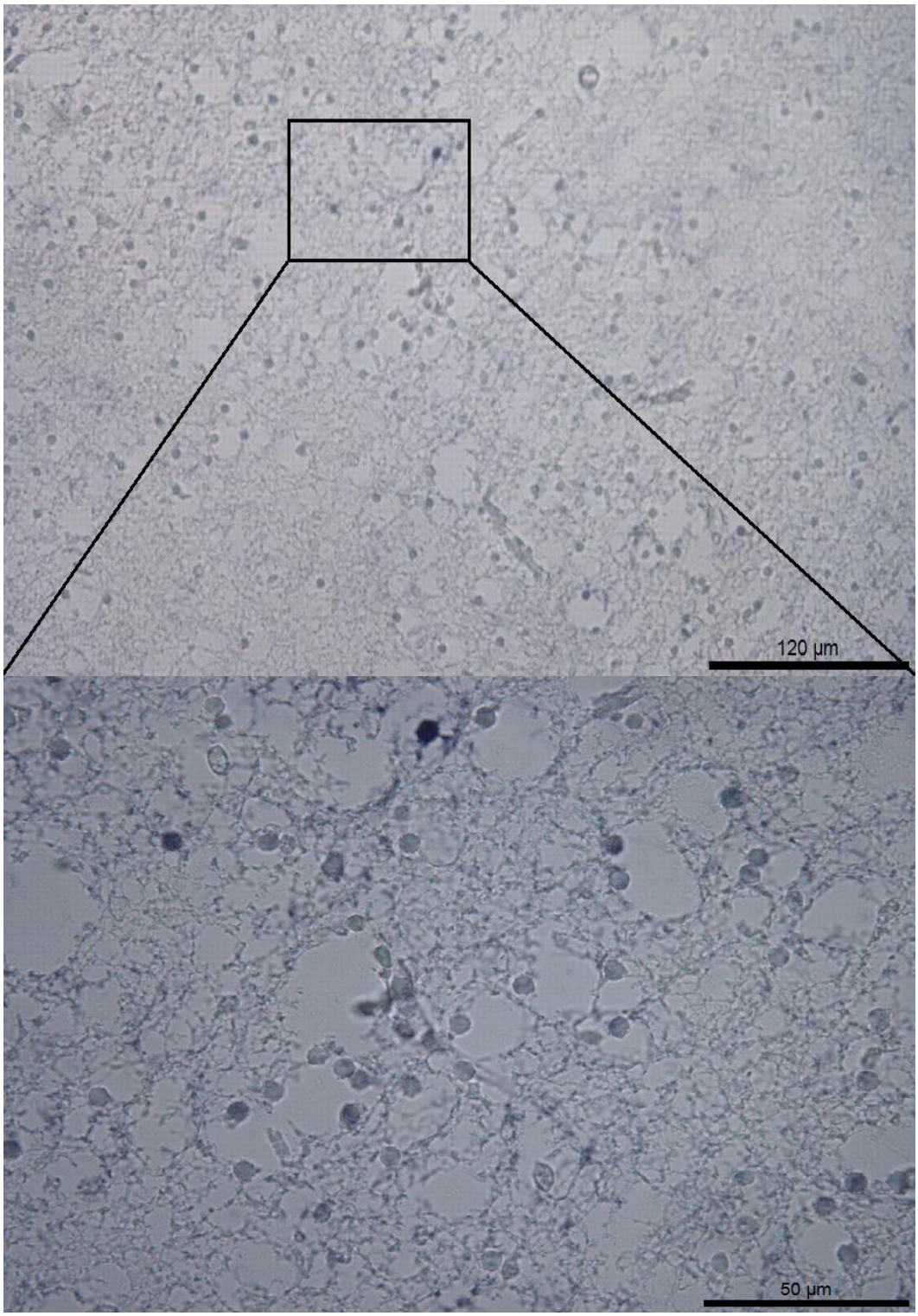


Figure 3.5. Chromogenic in situ hybridization localisation of CB_1 receptors in the <u>cerebellum</u> of human. The figure shows in situ hybridization images with high magnification (50X), which were obtained using a bright field microscope with a camera attachment.

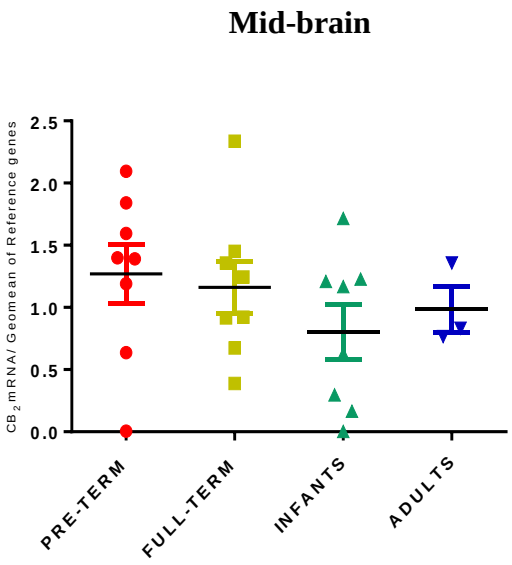
3.2.2 Changes in CB_2 receptor expression during postnatal development of the supraspinal centres

Although CB_2 mRNA was expressed in both the cerebellum and mid-brain at all ages there were no significant differences between the age groups tested (mid-brain: $F(3,23)=0.8885$, $P=0.4618$; one way ANOVA; Figure 3.6a), (cerebellum: $F(3,21)=0.4484$, $P=0.7211$; one way ANOVA; Figure 3.6b). Taqman RT-PCR results were showed that the expression of CB_2 receptors in mid-brain was more highly expressed in all age tested comparing with cerebellum.

The expression of CB_2 receptors within the mid-brain and cerebellum was also investigated using chromogenic in situ hybridization (n=4) in pre-term, full-term, infant and adult human brain. A number of studies were able to detect the presence of CB_2 in rodents and human brains (LU et al., 2000, Núñez et al., 2004). In situ hybridization expression of CB_2 receptors was found in the mid-brain, there were no statistically differences in expression of CB_2 receptor between the age groups tested ($F(3,11)=0.9154$, $P=0.2084$; one-way ANOVA; Figure 3.7 and 3.8).

Similar to the expression of CB_2 receptor in the mid-brain, CB_2 receptor in situ hybridization staining in the cerebellum did not change significantly between all aged tested ($F(3,11)=2.877$, $P=0.0895$; one-way ANOVA; Figure 3.9 and 3.10). In situ hybridization staining was shown that the expression of CB_2 in cerebellum was less expressed in all tested age in comparison with mid-brain. Moreover, the in situ hybridization expression pattern of CB_2 receptor was similar in both, mid-brain and cerebellum.

A)



B)

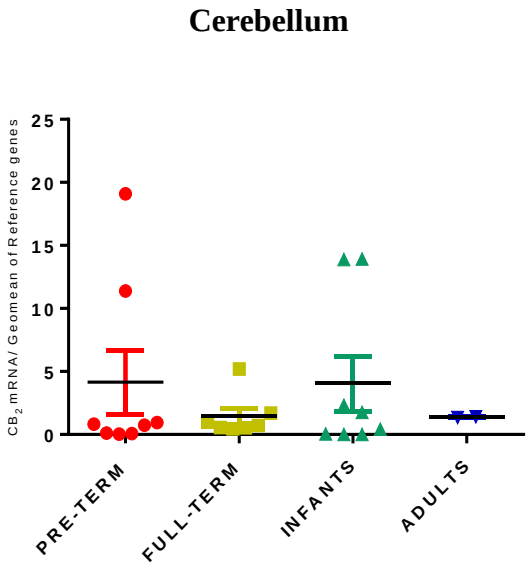
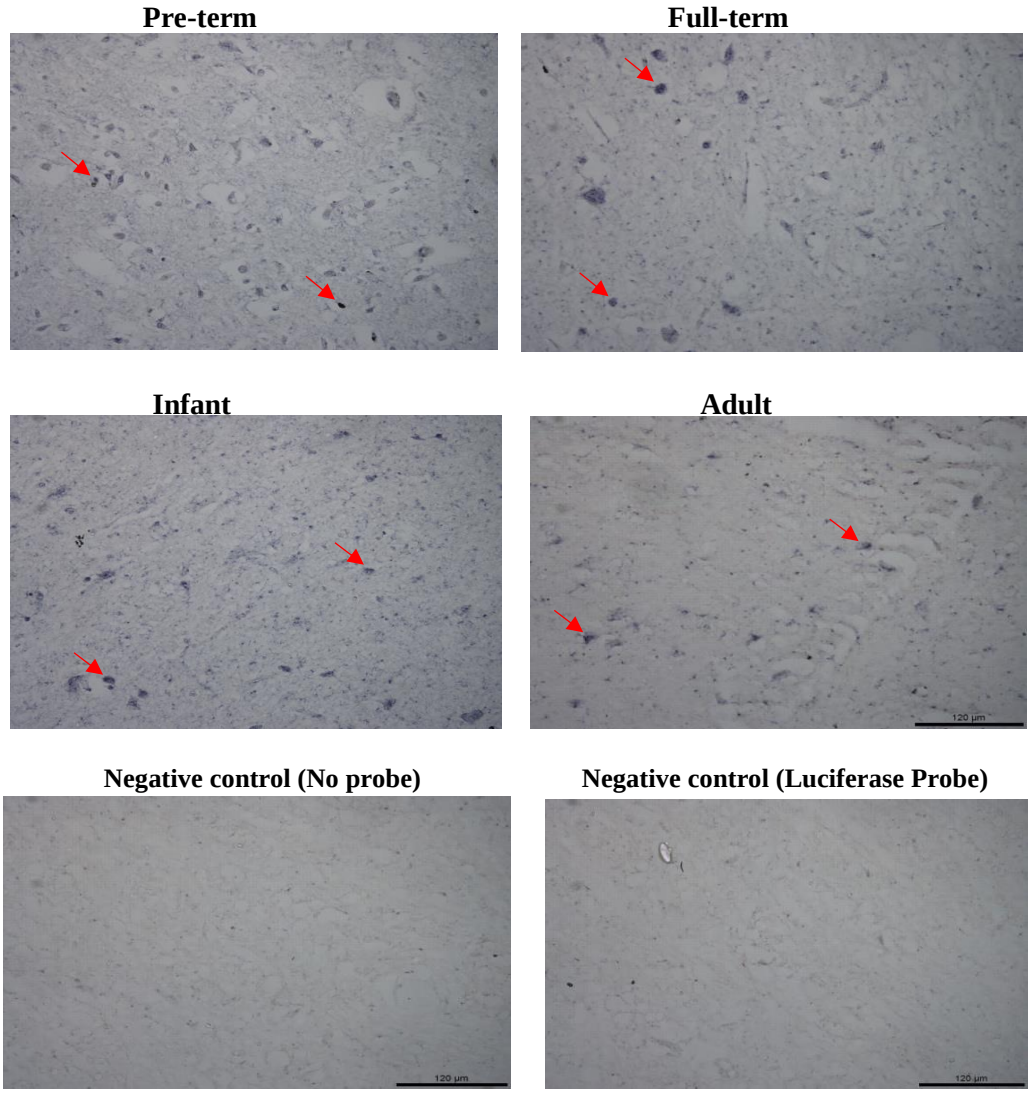


Figure 3.6. Taqman qPCR analysis of the expression of CB_2 receptor mRNA levels in (A) mid-brain and (B) cerebellum of pre-term, full-term, infant and adult human. CB_2 mRNA level in the mid-brain decreased not significantly as the brain aged, while no significant age-related differences were found in the level of CB_2 transcript levels in cerebellum. Data shown here represent mean ±SEM, one-way ANOVA.

A)



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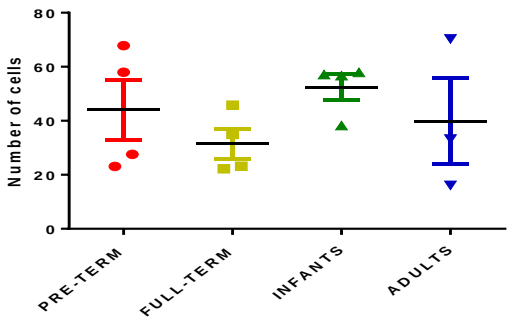


Figure 3.7. Chromogenic in situ hybridization localisation of CB_2 receptors in the <u>mid-brain</u> of pre-term, full-term, infant and adult human. (A) Upper panel shows in situ hybridization images (20X magnification), which were obtained using a bright field microscope with a camera attachment. (B) Lower panel shows quantification of in situ hybridization staining, cell counting were used where appropriate. The expression of CB_2 receptor in mid-brain did not change significantly over development. Data shown here represent mean ± SEM, one-way ANOVA with Bonferroni post-tests.

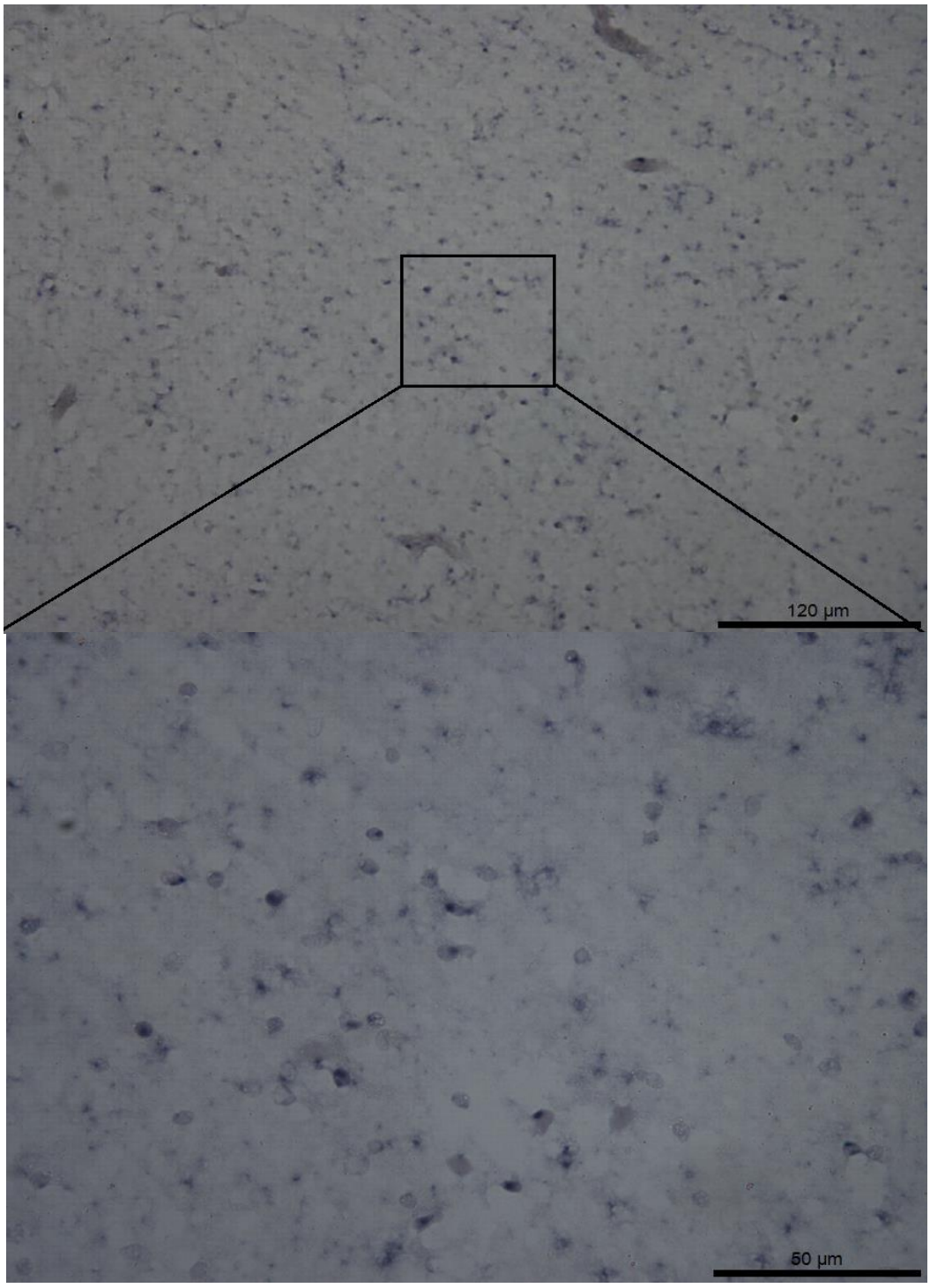
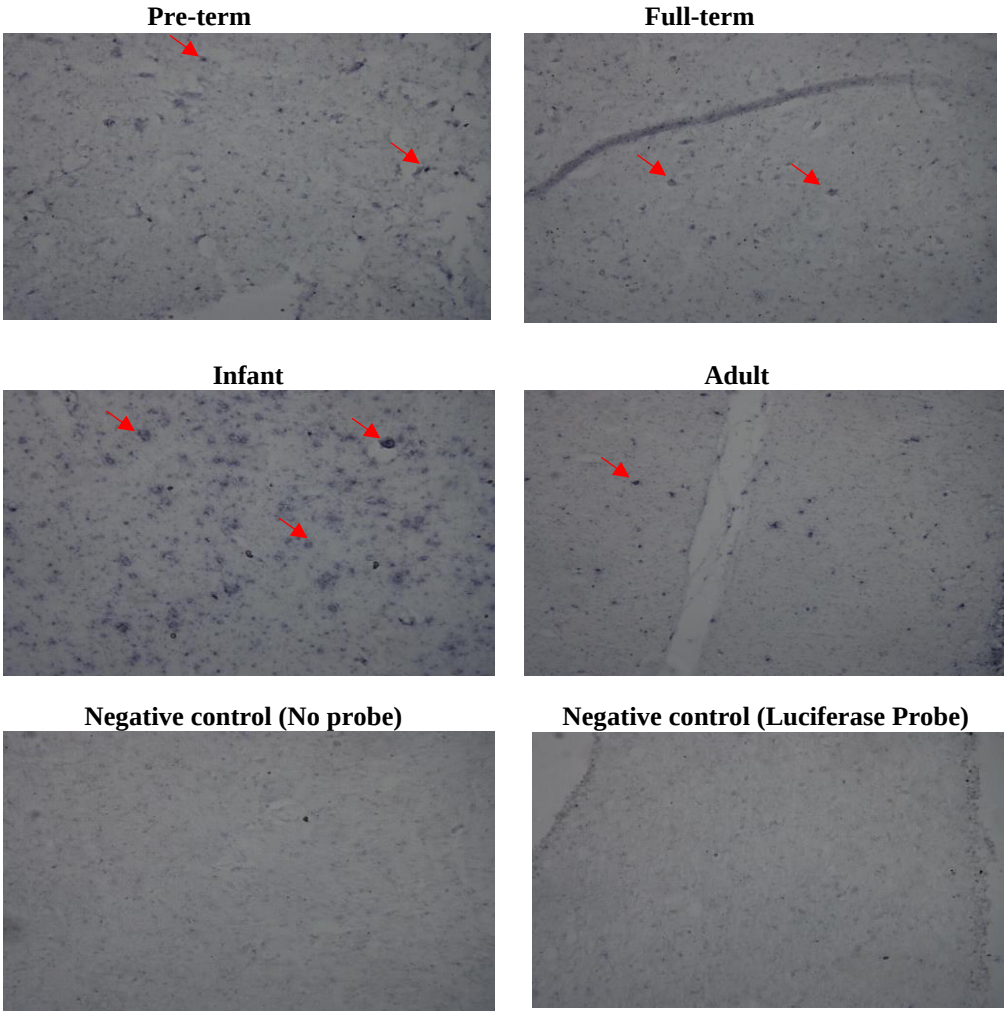


Figure 3.8. Chromogenic in situ hybridization localisation of CB_2 receptors in the <u>mid-brain</u> of human. (A) The figure shows in situ hybridization images with high magnification (50X), which were obtained using a bright field microscope with a camera attachment.

A)



B)

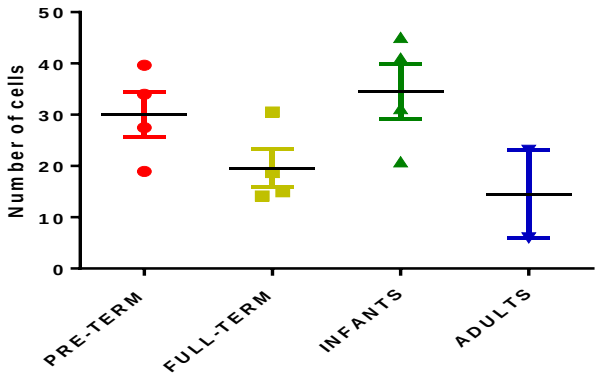


Figure 3.9. Chromogenic in situ hybridization localisation of CB_2 receptors in the <u>cerebellum</u> of pre-term, full-term, infant and adult human. (A) Upper panel shows in situ hybridization images (20X magnification), which were obtained using a bright field microscope with a camera attachment. (B) Lower panel shows quantification of in situ hybridization staining, cell counting were used where appropriate. The expression of CB_2 receptor in cerebellum did not change over development. Data represent mean ± SEM, one-way ANOVA.

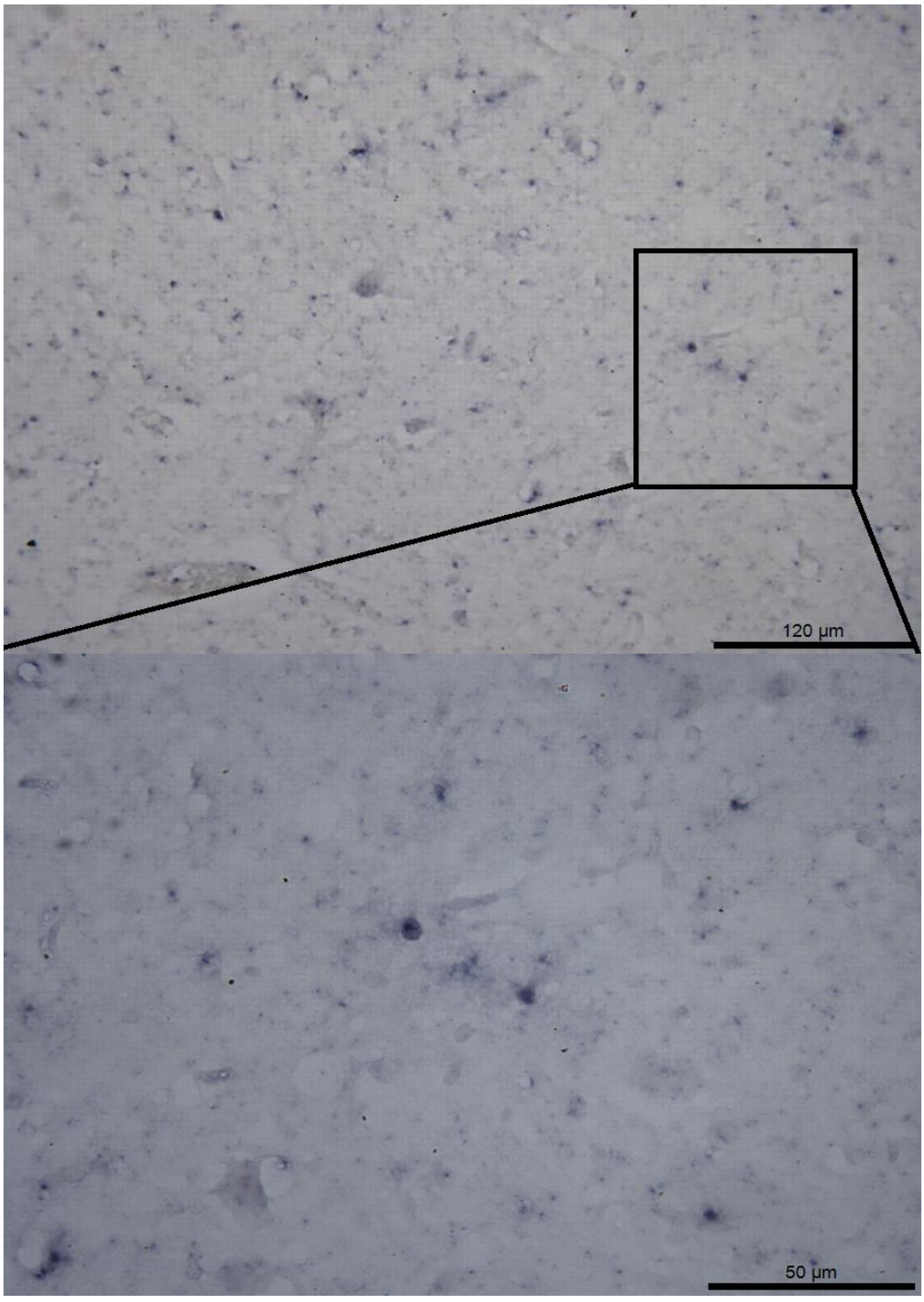
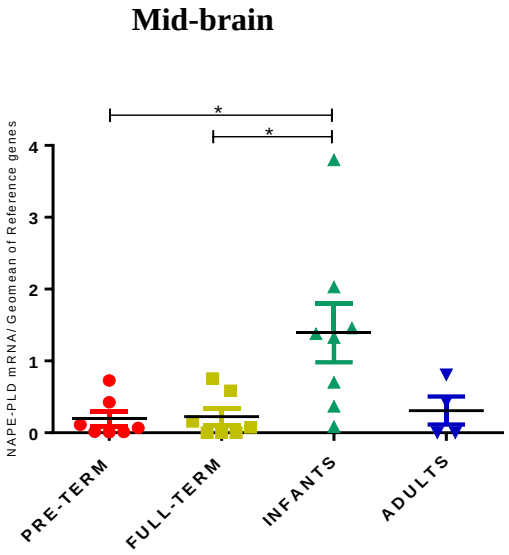


Figure 3.10. Chromogenic in situ hybridization localisation of CB_2 receptors in the <u>cerebellum</u> of human. (A) The figure shows in situ hybridization images with high magnification (50X), which were obtained using a bright field microscope with a camera attachment.

3.2.3 Changes in NAPE-PLD enzyme expression during postnatal development of the supraspinal centres

NAPE-PLD is an important enzyme involved in the production of one of the main endocannabinoid anandamide. As several reports suggested that anandamide levels increases with ageing (Long et al., 2012), an equivalent increase in the expression of NAPE-PLD was predictable. The expression of NAPE-PLD enzyme was investigated using Taqman RT-PCR. In line with previously published studies, NAPE-PLD mRNA levels in the mid-brain increased as the brain aged. NAPE-PLD mRNA transcript levels were higher in infant compared to both pre-term and full-term groups ($F(3,22)=5.024$, $P=0.0084$; one way ANOVA; infant vs. pre-term and infant vs. full-term; both $P<0.01$; Figure 3.11a). However, the expression of NAPE-PLD mRNA decreased in adult brain. Similar to the mRNA transcript level of NAPE-PLD in the mid-brain, NAPE-PLD mRNA transcript levels in the cerebellum peaked in infancy. RT-PCR data indicated that NAPE-PLD mRNA expression level was significantly increased in infant ($F(3,21)=4.813$, $P<0.05$; one-way ANOVA; Figure 3.11b).

A)



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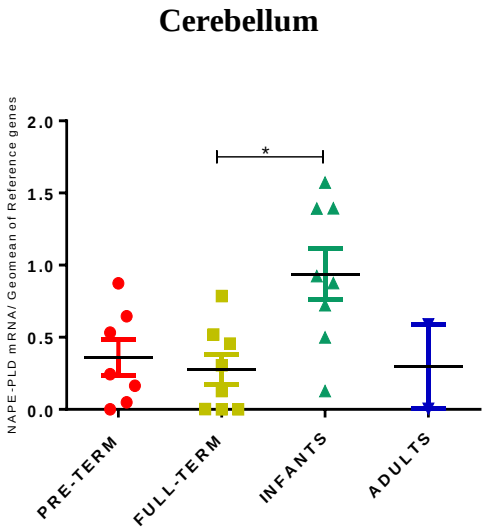
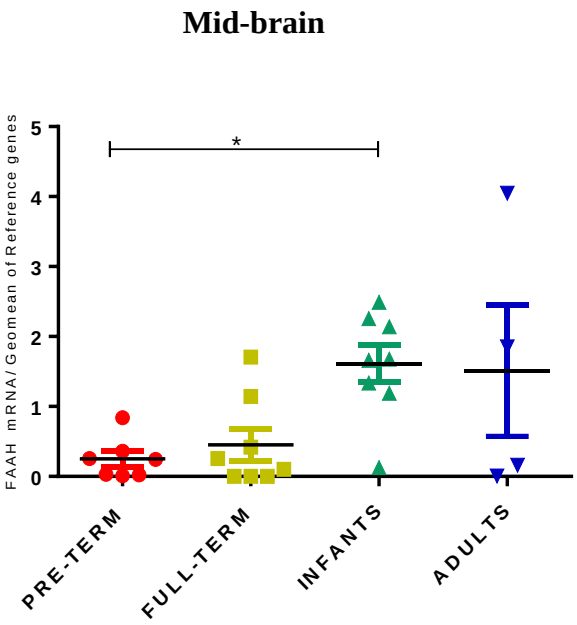


Figure 3.11. Taqman qPCR analysis of the expression of NAPE-PLD enzyme mRNA levels in (A) mid-brain and (B) cerebellum of pre-term, full-term, infant and adult human. NAPE-PLD mRNA transcript level in both midbrain and cerebellum peaked in infant and decreased in adult tissues. Data represent mean ±SEM. * = P<0.05, one-way ANOVA with Bonferroni post-tests.

3.2.4 Changes in FAAH enzyme expression during postnatal development of the supraspinal centres

FAAH is abundantly expressed throughout the CNS and neurons that express FAAH are found in close proximity to nerve terminals that also express the CB_1. This indicates a role of FAAH in the catalysis of AEA (Egertova et al., 2003, McKinney and Cravatt, 2005). In line with previously published studies, FAAH was expressed in the regions tested, mid-brain and cerebellum, using quantitative PCR. As shown in Figure 3.12a, the mRNA transcript levels of FAAH in mid-brain increased as the human brain aged. Data indicated that FAAH mRNA transcript levels were significantly increased in infant brains compared with pre-term brains ($F(3,21)=4.311$, $P<0.05$; one-way ANOVA; Figure 3.12b). FAAH mRNA transcript levels in cerebellum showed the same pattern except that in adult brains the mRNA expression level of FAAH return to baseline levels. The qPCR results showed that FAAH mRNA level was significantly higher in infant brains comparing with full-term brains ($F(3,21)=4.999$, $P<0.01$; one-way ANOVA) (Figure 3.12).

A)



B)

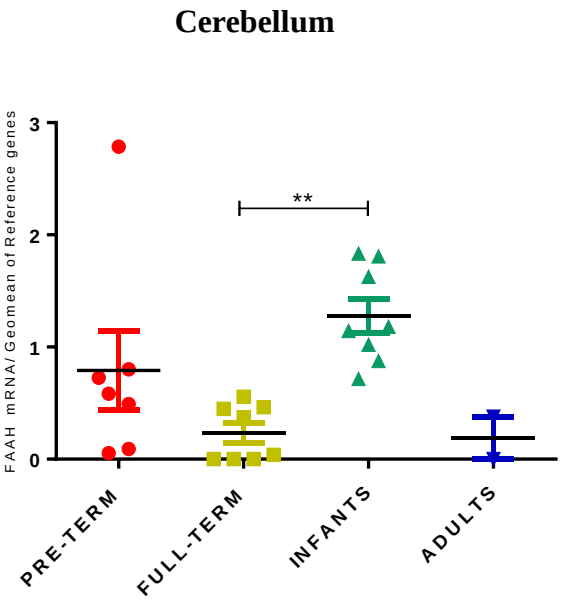


Figure 3.12. Taqman qPCR analysis of the expression of FAAH enzyme mRNA levels in the (A) mid-brain and (B) cerebellum of pre-term, full-term, infant and adult human. mRNA expression levels of FAAH in midbrain was increased as brain aged. However, FAAH mRNA transcript levels in cerebellum increased until infant then decreased strongly in adult cerebellum. Data represent mean ±SEM. *, ** = $P<0.05$ and $P<0.01$ respectively, one-way ANOVA with Bonferroni post-tests.

3.2.5 Expression of GPR55 receptor expression throughout postnatal development of the supraspinal centres

The expression of GPR55 receptors was investigated using Taqman RT-PCR technique. Ryberg et al. (2007) reported that GPR55 mRNA is found in different brain tissues such as cerebellum, hippocampus and brain stem, however the levels in all brain tissues are significantly lower than those for CB_1 receptors. For GPR55 qPCR experiments, efforts were made to detect any mRNA expression in human brain tissues. Taqman qPCR assay could not detect any expression for the GPR55 in human fixed brain tissues and commercially available RNA from human brain. In order to confirm that the primers and probe for GPR55 were functional, Taqman RT-PCR was carried out for commercial human spleen RNA, as a positive control for GPR55 mRNA expression, and GPR55 expression was detected in the commercial spleen RNA.

3.2.6 Expression of DAGLα receptor expression throughout postnatal development of the supraspinal centres

DAGLα is an enzyme important for the synthesis of 2-AG. There are several studies that described DAGLα distribution in different rat brain regions (Yoshida et al., 2006, Katona et al., 2006, Yoshida et al., 2011). Although efforts were made to detect DAGLα mRNA expression in tested human brain tissues, Taqman qPCR assay could not detect the expression of DAGLα in human fixed brain tissues, to test that the primers and probe for DAGLα are functional, Taqman RT-PCR was carried out for commercial human brain RNA. The Taqman assay was able to detect the expression of DAGLα in commercial human brain RNA.

3.3 Discussion

The endocannabinoid system is involved in the control of several central and peripheral functions, including neurotransmission, inflammation, hormone release, respiratory function and neural development (Pertwee, 2005, Pacher et al., 2006). Although the endocannabinoid system is an integral component of pain control (Starowicz et al., 2013), its role in the developing pain system in humans is not understood. In this study, the expression of different components of the endocannabinoid system in mid-brain and cerebellum in human brain was investigated.

The expression of endocannabinoid related mRNA levels were included in current study to investigate the regulation of the targets at transcriptional level. The targets including CB_1, CB_2, FAAH, NAPE-PLD, GPR55 and DAGLα were chosen to give a wide demonstration of this system. In this study we revealed postnatal changes in the mid-brain and cerebellum of human and these changes may have important functional values on pain modulation.

3.3.1 The expression of CB_1 within the mid-brain and cerebellum during postnatal development

The results of this study did not show significant changes in the expression of CB_1 mRNA levels with postnatal age in mid-brain. However, in situ hybridisation experiments displayed that the number of CB_1 positive cells in mid-brain was highest during infancy. On the other hand, peak CB_1 receptor mRNA expression levels was observed significantly at infant age by Taqman qPCR in cerebellum. The analysis of in situ hybridisation showed a trend but not significant that CB_1 receptor mRNA

expression peaks during infancy in cerebellum. These findings are consistent with studies showing similar trend from early life to adulthood, with CB_1 receptor mRNA decreased in adulthood in dorsolateral prefrontal cortex (Long et al., 2012). The reduced CB_1 receptor expression might be the result of reduction of the suppression of presynaptic neurotransmission that is mediated by CB_1 receptors (Long et al., 2012). Microinjection of morphine in basolateral amygdaloid nucleus produced analgesic effects, and CB1 reactivity is presence in GABAergic neurons in these area (Helmstetter et al., 1993, Tsou et al., 1998a). This suggests that analgesic potency is indirectly associated with CB_1 modulated GABAergic transmission neurons (Tsou et al., 1998a). Further investigation of the relationship between CB_1 and GABA activity in the pain pathways would be promising.

3.3.2 The expression of CB_2 within the mid-brain and cerebellum during postnatal development

CB_2 receptor is expressed extensively in peripheral tissues, particularly in the immune system. In addition, the recently reported expression of CB_2 receptor in the nervous system leads to questions about its potential role in the nervous system. Currently, the role of the CB_2 receptors in brain and pain system development is poorly understood. In line with previous published data in mouse hippocampus (Li and Kim, 2015), CB_2 receptor mRNA expression level did not significantly change with postnatal age using Taqman qPCR and in situ hybridisation in both mid-brain and cerebellum human tissues. Moreover, these results from human mid-brain and cerebellum are in accord with the stable developmental pattern of CB_2 receptor expression in the post-mortem human prefrontal cortex (Choi et al., 2012). CB_2 receptor mRNA are expressed mainly in microglia but it has very low expression in neurons and astrocytes (Zhang et al.,

2014). However, it still needs to be determined whether the expression of CB_2 receptor in individual cell types is also not changed during postnatal development.

3.3.3 The expression of NAPE-PLD within the mid-brain and cerebellum during postnatal development

The expression of NAPE-PLD mRNA in the mid-brain and cerebellum was highest in infant compared to both pre-term and full-term neonates, which is suggesting that expression of AEA increases within mid-brain region of human during postnatal development (Berrendero et al., 1999). Importantly, because of the nature of tissue in current study, early life inflammation could also affect the expression NAPE-PLD mRNA levels during infancy. This finding is extending to humans previous data of increased cortical NAPE-PLD expression levels from early life to infancy period (Morishita et al., 2005, Long et al., 2012), and further suggesting that AEA synthesis may be higher in adults rather than in neonates (Berrendero et al., 1999). One possible explanation of these findings is that AEA induces the depression of inhibitory synapses through TRPV1 receptor in developing mid-brain and it is possible that AEA facilitates the neurotransmission excitation in this region (Liapi and Wood, 2005, Grueter et al., 2010). Moreover, Kwok et al. (2017) reported that the data that obtained from liquid chromatography–mass spectrometry analysis exhibited significant increases in the levels of AEA in brain stem in rat during postnatal development; in parallel with this, the NAPE-PLD mRNA transcript levels in the PAG increased with aging.

3.3.4 The expression of FAAH within the mid-brain and cerebellum during postnatal development

The expression of the hydrolysing enzyme FAAH within the mid-brain and cerebellum was investigated using Taqman qPCR. FAAH mRNA expression levels steadily increased throughout postnatal development in the mid-brain. Previous study reported that the expression of the AEA hydrolysing enzyme FAAH increases during postnatal life in the hippocampus of rat (Morozov et al. (2004). Additionally, this data suggest that the amount of anandamide available for signalling at CB_1 receptors increases during the development of brain. On the other hand, the expression of FAAH enzyme mRNA fluctuated in cerebellum.

3.3.5 Conclusion

Overall, the data of the current study imply that human early postnatal life, infancy and adulthood are characterised by distinct differences in levels of cannabinoid receptors, synthesis and breakdown expression. A summary of all the major findings described in this chapter is included in Table 3.1. These findings show that endocannabinoid system is a promising target for pain management in immature patients. Activation of cannabinoid receptors in the mid-brain is anti-nociceptive regardless of postnatal age (Finn et al., 2003). The AEA synthesising enzyme NAPE-PLD became highly expressed especially at infancy, whereas the 2-AG synthesising enzyme DAGLα has undetectable mRNA levels over postnatal development. It would be beneficial to include MAGL, an enzyme that terminates 2-AG activity, in order to fully describe the development of endocannabinoid metabolic enzyme across postnatal life. In this study, GPR55 has undetectable expression mRNA levels in human mid-brain and cerebellum and these findings are inconsistent with the data that obtained

from rat brain (Kwok et al., 2017), which showed that the GPR55 mRNA transcript detected in rat brainstem nuclei. While different conditions surrounding death and variable post-mortem intervals are restrictions of using human brain materials, these factors did not appear has the main effect on endocannabinoid system mRNAs, but the shortage in the availability of human brain tissue from Nottingham biobank has the major obstacle to continue working on this project, so we changed our plan to investigate the age-related changes in brain and spinal cord microglia over development in next chapters.

Table 3.1. Age-related changes in the cannabinoid signalling system are illustrated, anatomical differences between the ages within the regions (mid-brain and cerebellum) are described.

	Pre-term	**Full-term**	**Infant**	**Adult**
Mid-brain	Expression of CB_1 receptors and NAPE-PLD are low, but high for CB_2 receptors	Expression of CB_1 receptors decrease, whereas FAAH increase	High levels of endocannabinoid synthesising and degrading enzymes (NAPE-PLD and FAHH respectively)	Expressions of CB_1 and CB_2 receptors are low, but high for FAAH
Cerebellum	Expressions of CB_1, NAPE-PLD and FAAH are low, but high for CB_2	Expression for FAAH increase, whereas CB_1 receptors increase	High levels of CB1 receptors, NAPE-PLD and FAAH	Expressions of CB_1 and CB_2 receptors, NAPE-PLD and FAAH are lowest

4 Chapter Four: Age-related changes in the phenotype and function of spinal cord and brain microglia

4.1 Introduction

Developmental and ageing studies suggest that microglia change over the lifespan. Neonatal microglia are involved in remodelling of the developing brain by improve the survival of neuron and phagocytose the cellular debris (Wake et al., 2009, Bilimoria and Stevens, 2015). In the adult brain, microglia are involved in local immune surveillance (Nimmerjahn et al., 2005), as well as maintenance of CNS homeostasis and may contribute to the neuronal network by helping in synaptic remodelling (Wake et al., 2009, Frost and Schafer, 2016). Previous studies have revealed that pain signalling in young animals is immature: nociceptive withdrawal thresholds are lower, and response magnitudes are higher during early postnatal period (Andrews and Fitzgerald, 1994, Fitzgerald and Jennings, 1999, Fitzgerald, 2005). Microglia expression of pro-inflammatory cytokines including IL-1β and TNF-α increase with aging; this elevation is accompanied by a decrease in expression of anti-inflammatory cytokines, such as IL-10 and IL-4 (Ye and Johnson, 2002, McKelvey et al., 2015). These changes in inflammatory status are supposed to contribute to the chronic inflammatory conditions such as neurodegenerative diseases.

Microglial function is important for maintaining the CNS compartments in both health and disease, with a wide range of functional states that reveals the various roles of these cells (Askew and Gomez-Nicola, 2017). During CNS development, they prune the excess neuronal connections, remove dying cells and debris through phagocytosis, control synapses and monitor neurogenesis. Additionally, under normal physiological conditions, the microglia populations are maintained by self-renewal of resident cells that divide in situ and/or cells that are recruited from the circulating monocyte pool through an intact BBB and differentiate into microglia (Lawson et al., 1992,

Hashimoto et al., 2013). On the other hand, in CNS disorders they are the main regulators of the neuro-immune response and inflammation within CNS (Gomez-Nicola and Perry, 2015).

The role of glial cells in the development and maintenance of neuropathic pain in adults is known and is controlled by a pro-inflammatory response that results in sensitization of neurons in the DH (Gao and Ji, 2010, Taves et al., 2013). However, neuropathic pain is not observed following early life peripheral nerve injury in both humans (Howard et al., 2014) and animals (Moss et al., 2007, Hathway et al., 2009a, Vega-Avelaira et al., 2012). Nerve injury in early life causes little elevation in the expression of either microglia (Iba-1, integrin-α) or astrocytes (GFAP) markers in the DH (Moss et al., 2007, Vega-Avelaira et al., 2007) and no increase in pro-inflammatory cytokines such as TNF in the dorsal horn 7 days following injury (McKelvey et al., 2015). In addition, after nerve injury in infant the expression of anti-inflammatory factors such as GATA3 is increased, whereas there is a limited pro-inflammatory response relative to adults (McKelvey et al., 2015). The molecular changes that occur during development from neonate to adult, which influence pro- and anti-inflammatory microglial phenotype is not well understood.

Martinez and Gordon (2014) explained the limitations of M1/M2 macrophage polarization hypothesis, providing a perspective that challenges the possibility of applying this frame to microglial cells. On the basis of observations from recent studies (Chiu et al., 2013, Yamasaki et al., 2014, Wes et al., 2016, Morganti et al., 2016), pro-inflammatory (M1) and anti-inflammatory (M2) states fail to emerge as isolated pure phenomena *in vivo*. In addition, macrophage activation was studied in

the context of monocyte or bone-marrow-derived macrophages. However, microglia are resident CNS macrophages, originating from extraembryonic yolk sac, different from the circulating hematopoietic system (Schulz et al., 2012) and highly adapted to their environment (Link et al., 2015). Nowadays, terminology suggesting established important pathways of microglial activation hinders hybrid pro- and anti-inflammatory cytokines profile (Ransohoff, 2016, Askew and Gomez-Nicola, 2017). Microglial cells are usually stimulated by PAMPs and DAMPs, and secrete multiple inflammatory cytokines, ROS and NO (Block et al., 2007). PAMPs are recognized by microglial cells through TLRs that activate many signalling pathways, including NF-kB which the most distinctive (Bianchi, 2007). Whilst DAMPs such as HMGB1 and ATP which are released by necrotic cells, bind to specific receptors expressed on microglia such as RAGE and P2X4, respectively (Hori et al., 1995).

Aims

In this chapter, the functional phenotype of neonatal and adult microglia isolated from rat brain and spinal cord were evaluated. Most studies of microglia have been performed using neonatal primary microglia isolated from the cerebral cortex as they are easier to isolate and culture than primary adult microglia. However, the phenotype of neonatal and adult microglia differs and data obtained from neonatal cells cannot necessarily be extended to the adult. In addition, the ability of microglia to survey their microenvironment, may differ with age (Nimmerjahn et al., 2005). The overall aim in this chapter was to understand the global molecular basis for the potential phenotypic alteration of microglia over the postnatal period. To address the overall aim, we tested the hypothesis that microglial transcriptional profiles of inflammation are regionally heterogeneous and we postulated that ageing influences microglial inflammatory

phenotype in a region-dependent manner. To examine the above hypotheses, we established and validated a method to efficiently and consistently isolate microglia from neonatal and adult rat brain and spinal cord. Secondly, we investigated the differential expression profiles of microglia from brain and spinal cord. Subsequently, we determined the effect of PAMPs and DAMPs on the neonatal and adult microglial inflammatory transcriptome.

4.2 Results

4.2.1 Purity of isolated microglia in culture

4.2.1.1 Yield and purity of neonatal primary microglia

The method described in section 2.4.1 produced high purity primary microglia cultures from neonatal rat brain and spinal cord tissues. Immunocytochemistry for microglia specific marker Iba1, indicated that microglia cultures are >90% pure (Figure 4.1, 4.2). In addition, staining for cell-specific markers for astrocytes (GFAP), oligodendrocytes (CC1) and neurons (NeuN) demonstrates minimal contamination (Figure 4.1, 4.2). Few astrocytes are present in brain and spinal cord microglia cultures (4.05±0.35% and 3.45±0.78% respectively GFAP positive; Figure 4.1, 4.2). No oligodendrocytes were observed in the microglia cultures (Figure 4.1, 4.2), whereas very few NeuN immunopositive cells were present. The brain microglia isolation was yield 1.5 x 10^6 cells/brain and spinal cord microglia isolation yield was 1.1 x 10^6 cells/cord.

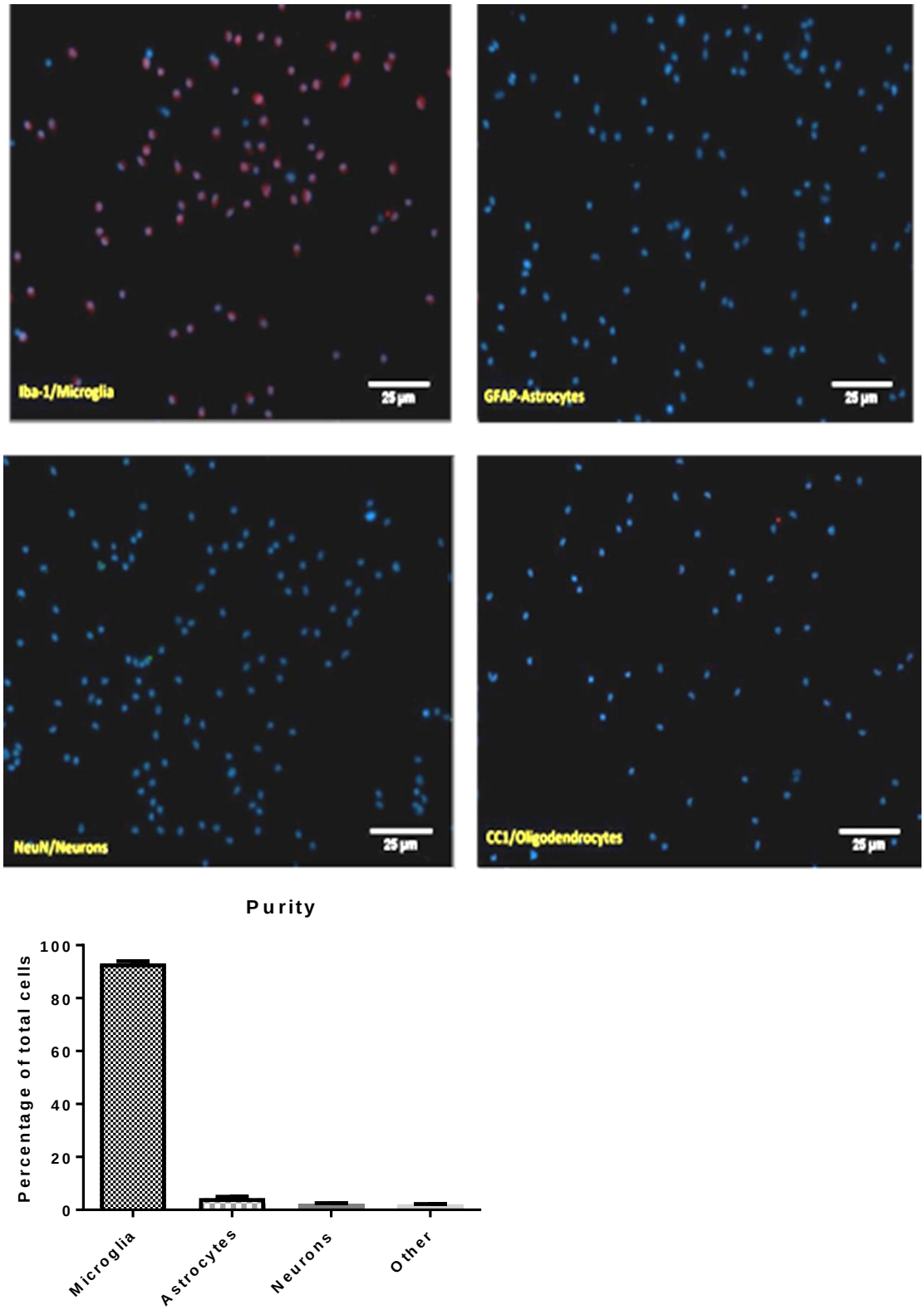


Figure 4.1. Isolation of high purity microglia from neonatal rat spinal cord as confirmed by immunocytochemistry analysis using microglia (Iba-1, red), astrocyte (GFAP, green), neuron (NeuN, green) and oligodendrocyte (CC1, red) specific markers. Staining of nuclei by DAPI (blue) is also shown. The graphs represent means±SEM obtained from three independent experiments.

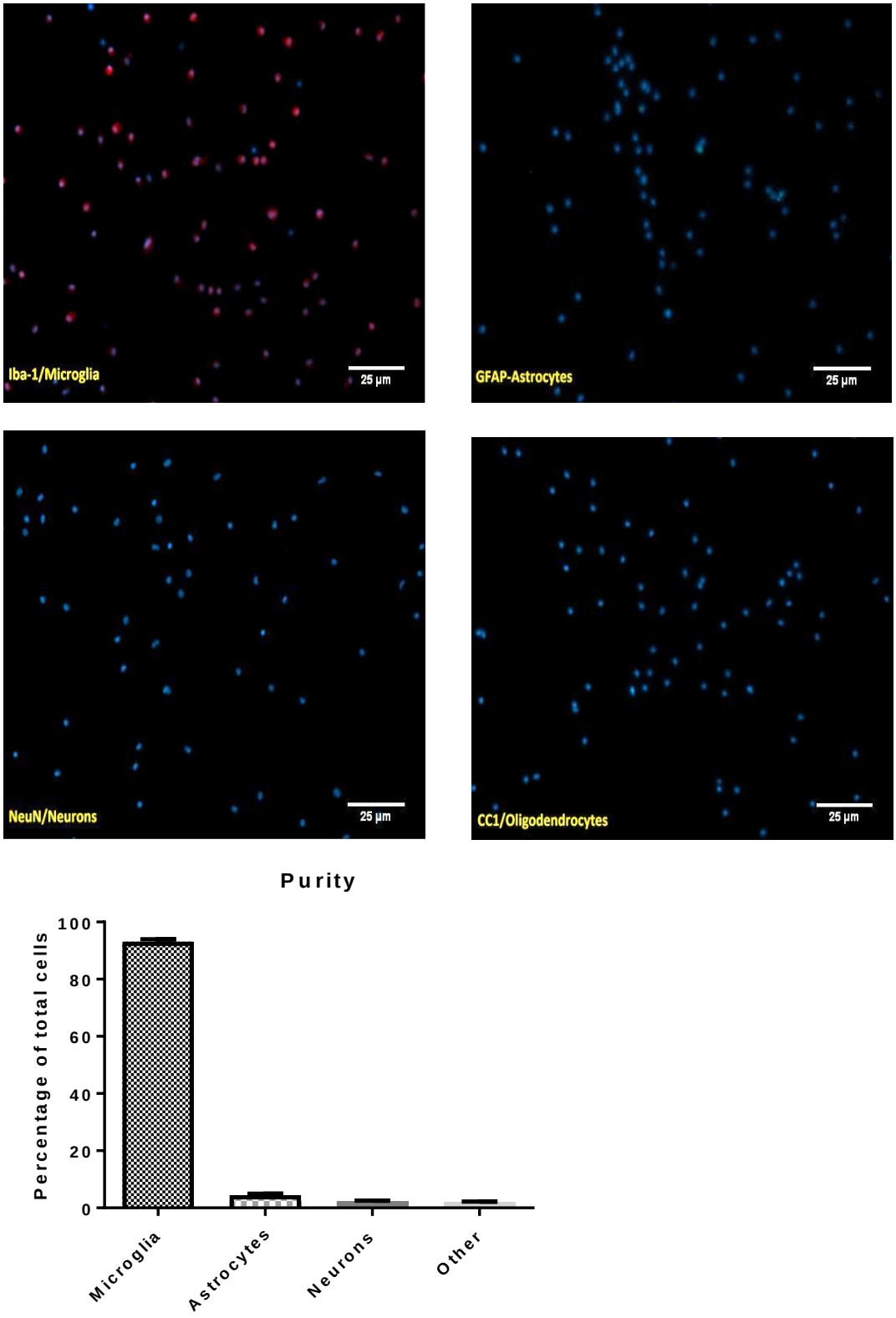


Figure 4.2. Isolation of high purity microglia from neonatal rat brain as confirmed by immunocytochemistry analysis using microglia (Iba-1, red), astrocyte (GFAP, green), neuron (NeuN, green) and oligodendrocyte (CC1, red) specific markers. Staining for nuclei of cells by DAPI (blue) is also shown. The graphs represent means±SEM obtained from three independent experiments.

4.2.1.2 Yield and purity of adult primary microglia

Adult primary microglia isolation from adult rat brain and spinal cord tissues resulted in high-purity cultures. As demonstrated by immunocytochemistry for microglia cell specific marker (Iba1), plated microglia cultures are >90% pure (Figure 4.3 and 4.4). Additionally, the extent of astrocytic contamination in microglia cultures was evaluated by determining the immunocytochemical staining for the marker GFAP (Figure 4.3, 4.4). Few astrocytes are present in brain and spinal cord microglia cultures (4.05%±0.51 and 4.15%±0.43 respectively GFAP positive; Figure 3, 4). Furthermore, the extent of oligodendrocyte and neuronal contamination in microglia cultures was evaluated using specific markers CC1 and NeuN, respectively. No oligodendrocytes or neurons were observed in the microglia cultures (Figure 4.3, 4.4).

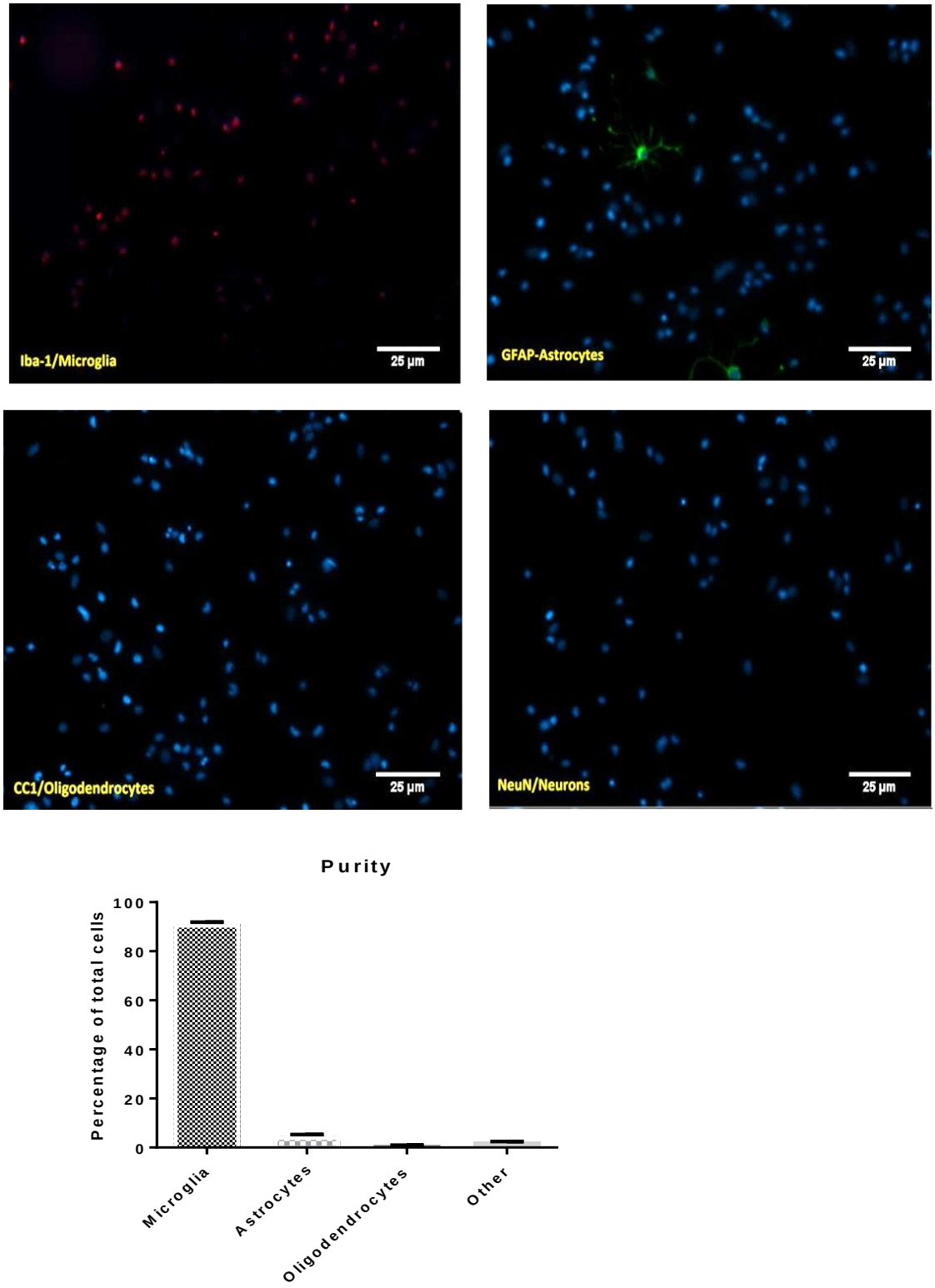


Figure 4.3. Isolation of adult spinal cord microglia culture purity. A culture purity of microglia isolation was determined via immunocytochemistry using markers specific markers for microglia (Iba1), astrocytes (GFAP), oligodendrocytes (CC1) and neurons (NeuN). Staining for nuclei of cells by DAPI (blue) is also shown. The graphs represent means±SEM obtained from three independent experiments.

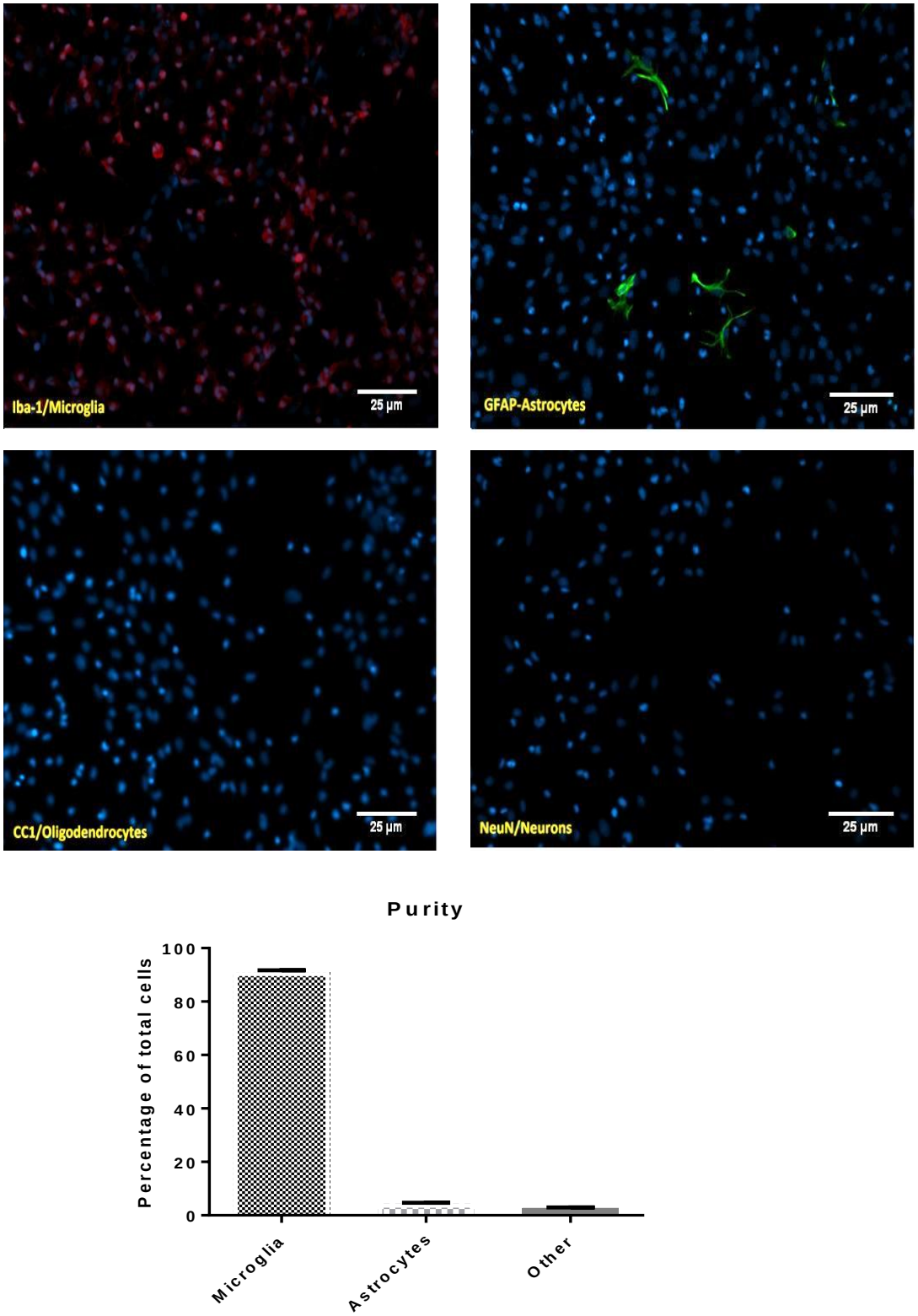
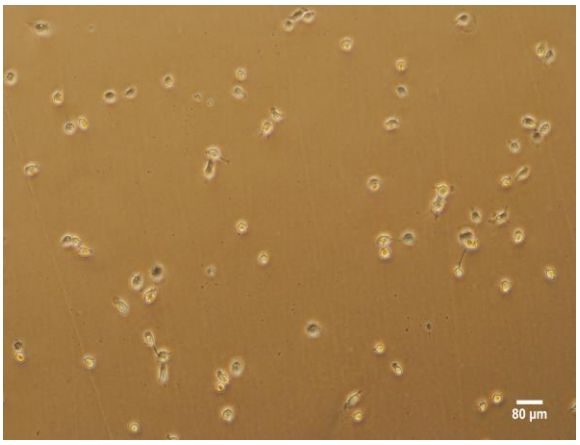


Figure 4.4. Isolation of adult brain microglia culture purity. Culture purity of microglia isolation was determined via immunocytochemistry using markers specific markers for microglia (Iba1), astrocytes (GFAP), oligodendrocytes (CC1) and neurons (NeuN). Staining for nuclei of cells by DAPI (blue) is also shown. The graphs represent means±SEM obtained from three independent experiments.

4.2.2 Microglial morphology

Neonatal and adult spinal microglia exhibit morphological differences; neonatal cells typically possess little cytoplasm and small or nonexistent processes. These cells possessing an amoeboid/phagocytic morphology (Figure 4.5A). However, adult cells having markedly more complex process and cytoplasmic materials, these ramified morphology facilitates the surveillance microglial function at adulthood (Figure 4.5B).

A)



B)

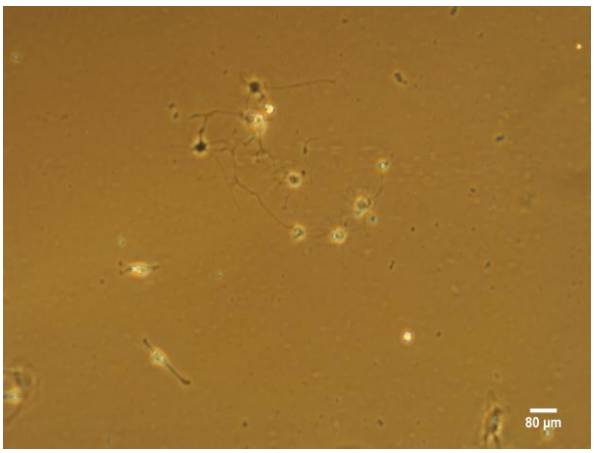


Figure 4.5. Morphological assessment of isolated neonatal and adult microglia. Phase contrast microscopy was used to study microglial morphology. In isolated neonatal microglia (A) significant morphological differences were observed compared to adult microglia (B).

4.2.3 Microglial developmental marker

To confirm that the observed differences in morphology were reflective of the stage of microglial development, the expression of **t**he transcription factor Sall1, which is most predominantly expressed in adult microglia (Matcovitch-Natan et al., 2016, Buttgereit et al., 2016, Koso et al., 2016) was assessed. Taqman qPCR results revealed that the Sall1 was expressed at significantly higher levels in adult microglia (15-fold greater than in neonatal cells) (Figure 4.6).

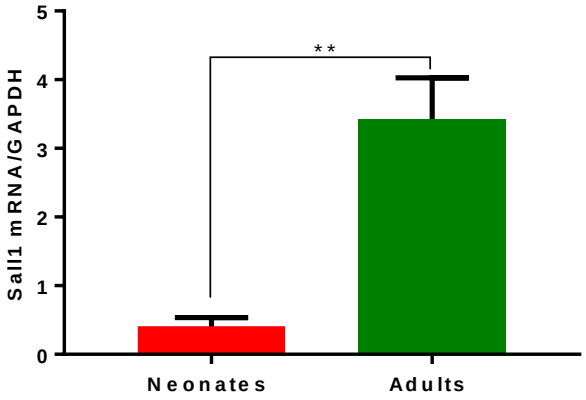


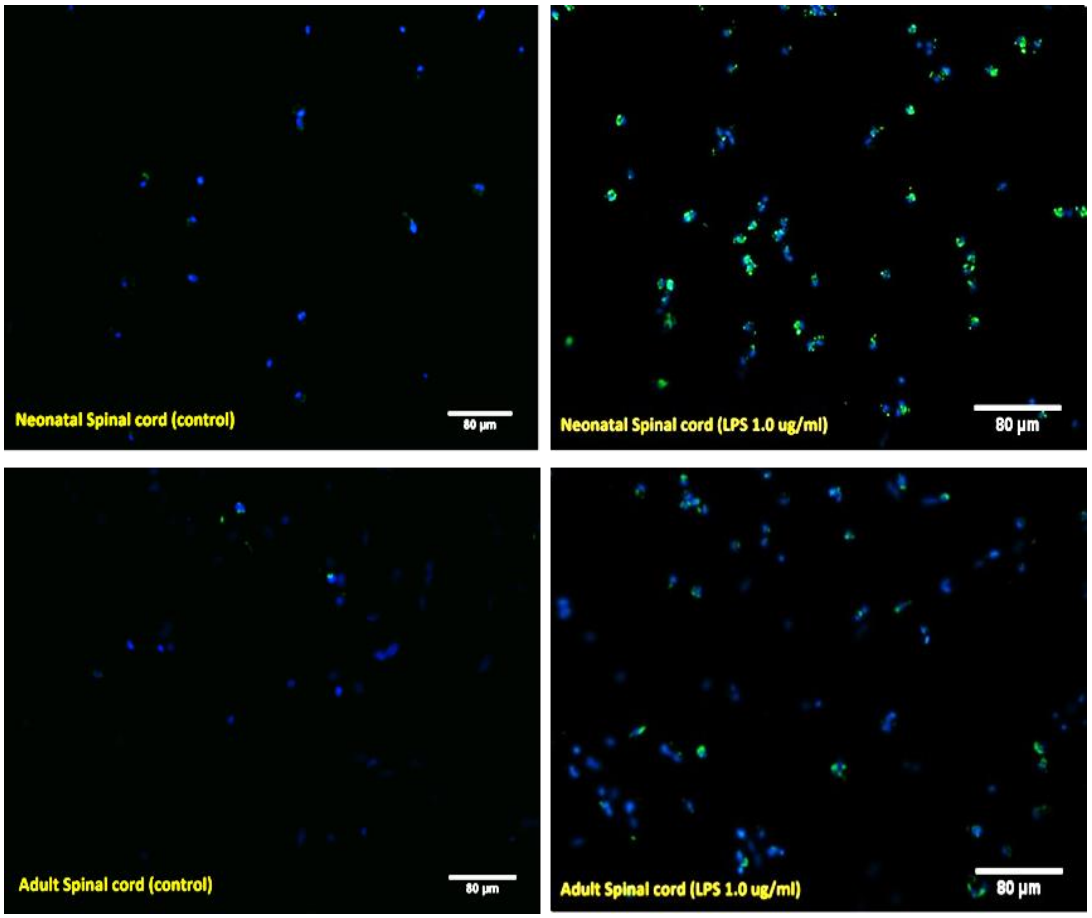
Figure 4.6. Sall1 gene expression in neonatal and adult microglia. Gene expression of Sall1 was measured by Taqman qPCR and normalized to GAPDH expression. The graphs represent means±SEM obtained from three independent experiments. Data were analysed by t-test. *, ** = P<0.05 and P<0.01 respectively.

4.2.4 Microglial phagocytosis

4.2.4.1 Spinal microglia phagocytosis

The phagocytic potential of microglia was evaluated by a phagocytosis fluorescence bead assay as described in materials and methods chapter (Section 2.6.). Compared to adult microglia, neonatal cells have more phagocytic activity when unstimulated (neonatal: 0.137±0.02 vs adult: 0.023±0.003; P=0.0596) and they increased phagocytic activity significantly following activation with LPS (neonatal: 0.323±0.03 vs adult: 0.174±0.027 $P<0.05$) see Figure 4.7A and B. As shown in Figure 4.8, ATP was capable of significantly stimulating phagocytosis in neonatal microglia (unstimulated: 0.137±0.013 vs stimulated: 0.264±0.039; $P<0.05$) but not in adult microglia (unstimulated: 0.067±.004 vs stimulated: 0.091±0.022; P=0.3423). Taken together, these results show that microglial phagocytic activity changes with age both at baseline and after LPS and ATP stimulation in a region-specific manner.

A)



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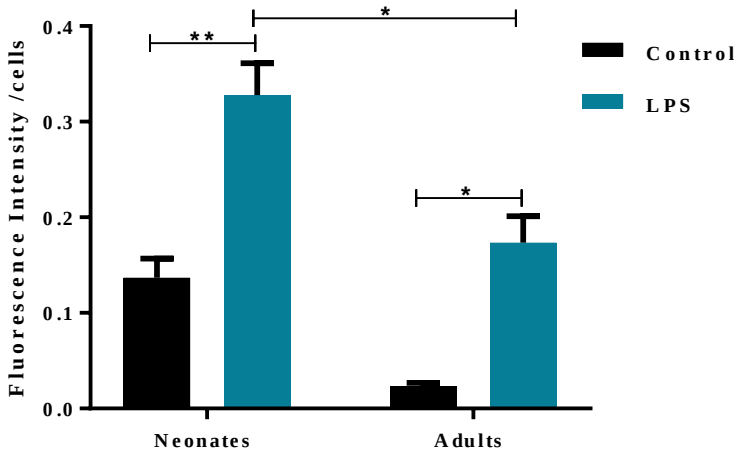
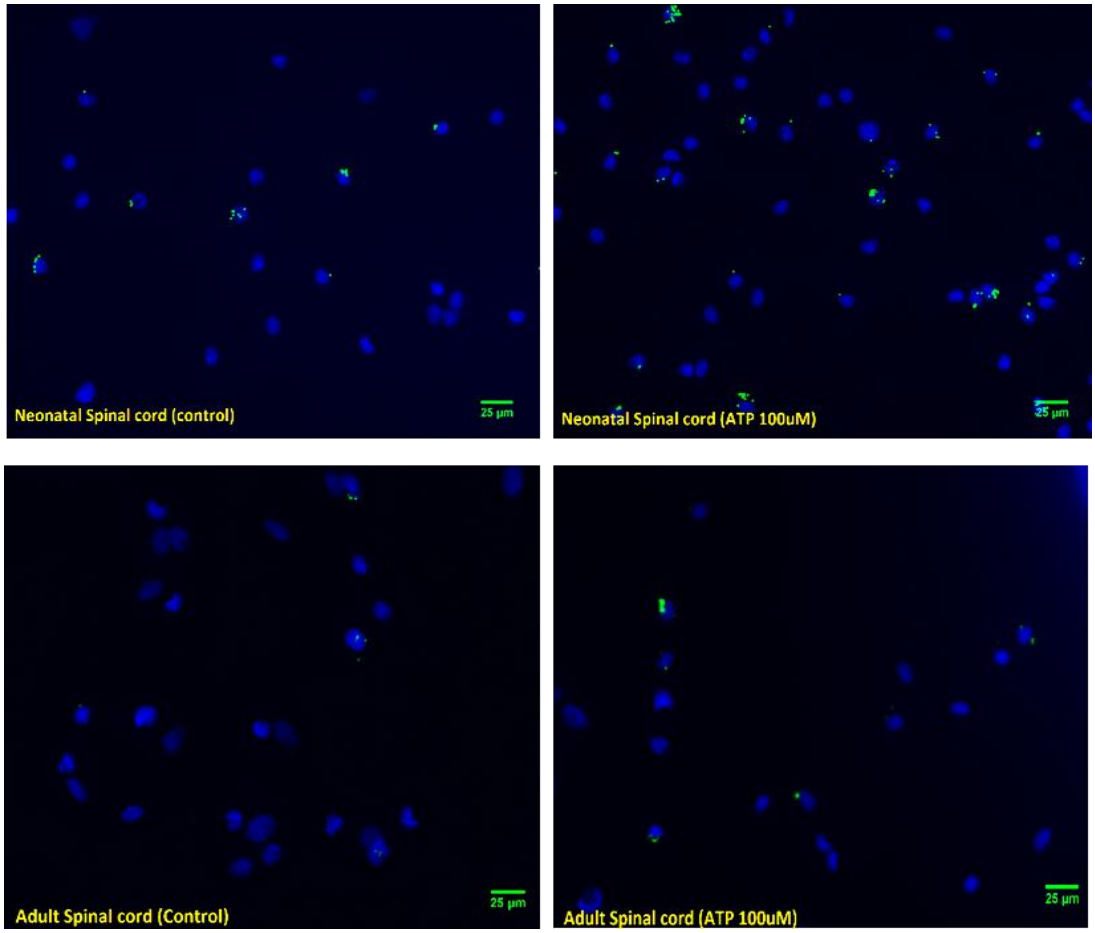


Figure 4.7. Microglia phagocytic activity in response to LPS in spinal cord. Primary microglial cells were incubated with fluorescent beads (green) for 2 hours then incubated at 37˚C with medium then fixed and stained with DAPI (blue). Primary cells treated with 100ng/ml LPS. A) Images show the phagocytic cells in primary neonatal and adult spinal cord rat microglia. B) Histogram of fluorescence intensity /number of total cells. The graphs represent means±SEM obtained from three independent experiments (4 slides for each group and quantify 4 fields from each slide). Data were analysed using student's t-test. *, **, *** = P<0.05, P<0.01 and P<0.001 respectively.

A)



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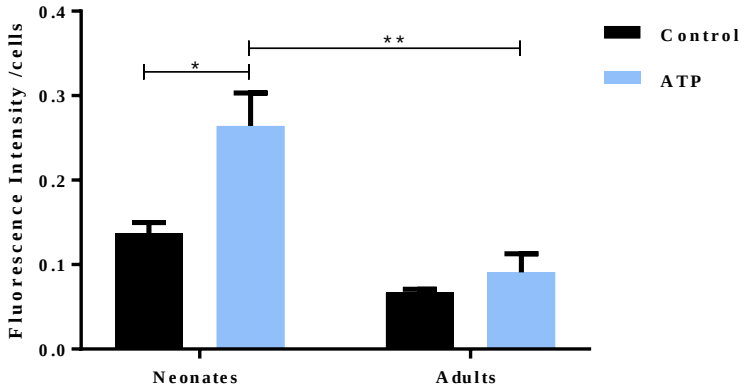
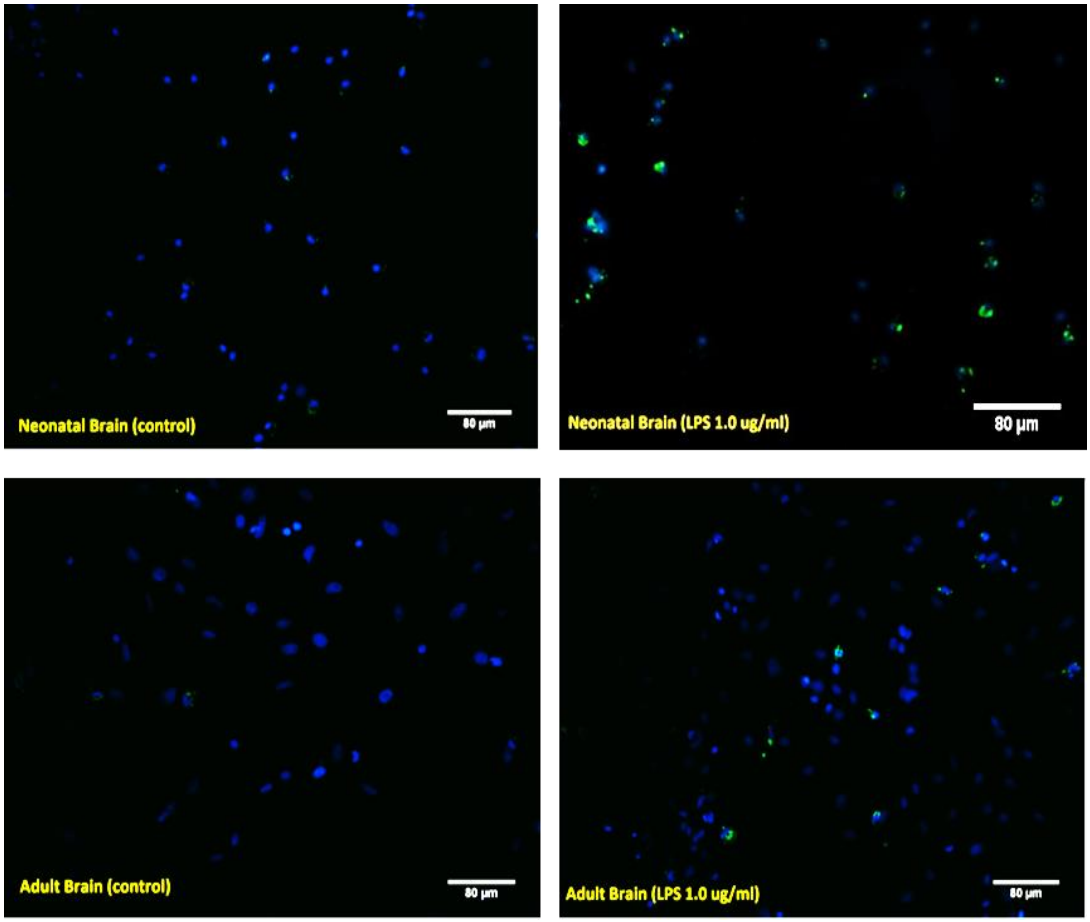


Figure 4.8. Microglia phagocytic activity in response to ATP in spinal cord. Primary microglial cells were incubated with fluorescent beads (green) for 2 hours then incubated at 37˚C with medium then fixed and stained with DAPI (blue). Primary cells treated with 100µM ATP. A) Images show the phagocytic cells in primary neonatal and adult spinal cord rat microglia. B) Histogram of flourescence intensity /number of total cells. The graphs represent means±SEM obtained from three independent experiments (4 slides for each group and quantify 4 fields from each slide). Data were analysed using student's t-test. *, **= $P<0.05$ and $P<0.01$ respectively.

4.2.4.2 Brain microglia phagocytic activity

Compared to adult microglia, neonatal cells have increased phagocytic activity when unstimulated (neonatal: 0.133±0.014 vs adult: 0.028±0.008; P<0.001) and following activation with LPS (neonatal: 0.215±0.022 vs adult: 0.053±0.003 P<0.0001) (see Figure 4.9). The phagocytic activity increased in both neonatal and adults microglia following ATP exposure, this was not statistically significant (neonates: unstimulated: 0.118±0.013 vs stimulated: 0.170±0.028; P=0.1762; Adults: unstimulated: 0.073±.023 vs stimulated: 0.124±0.011; P=0.1187) (Figure 4.10A and B).

A)



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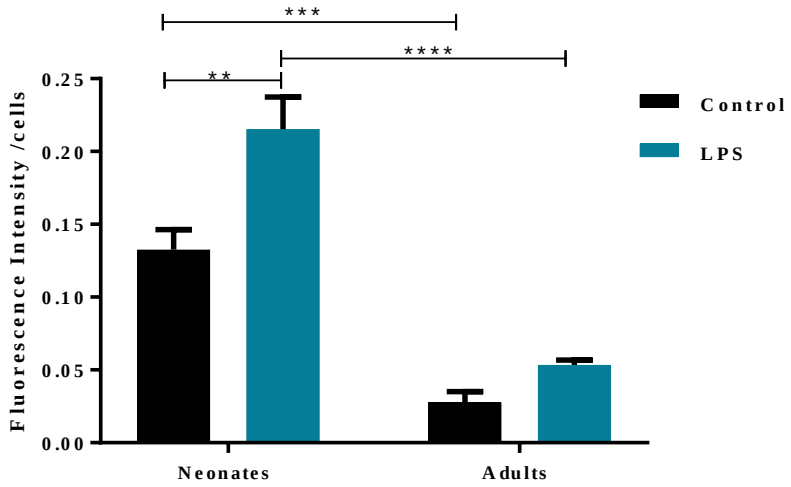
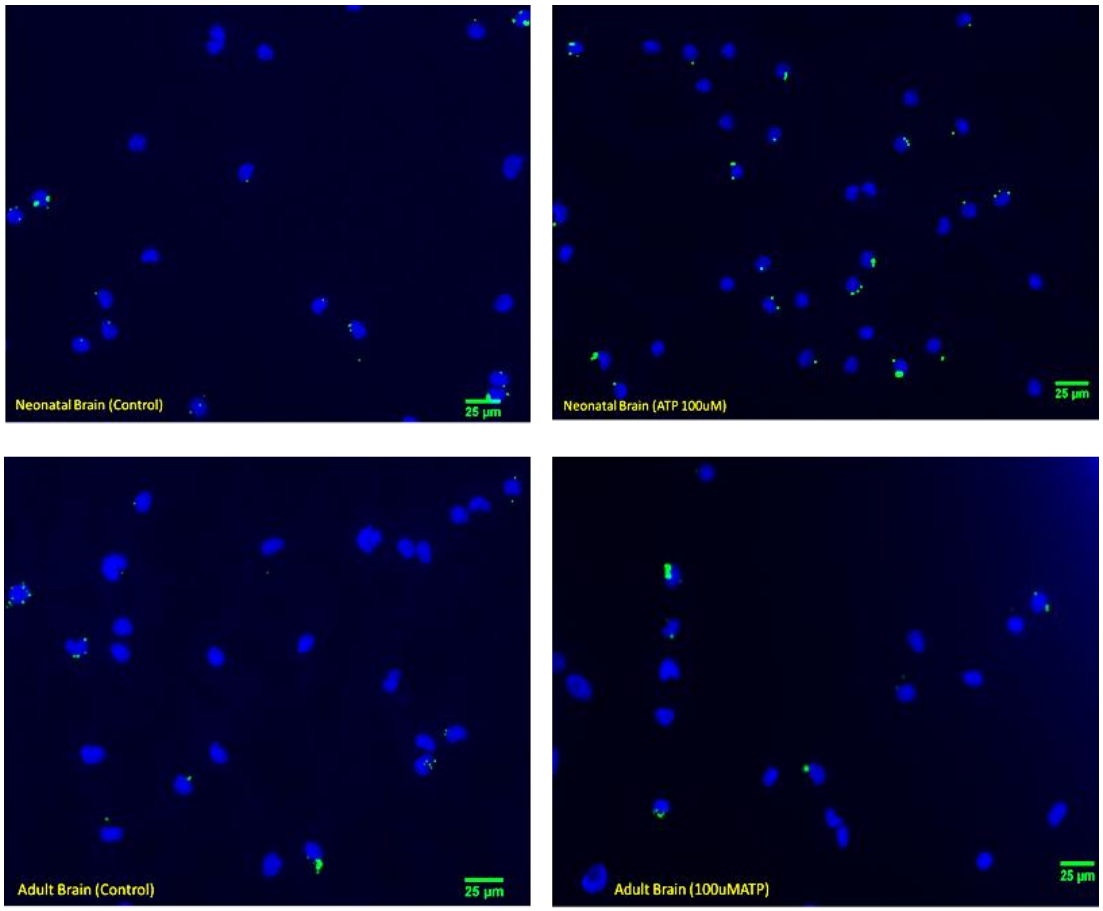


Figure 4.9. Microglia phagocytic activity impairment with age in response to LPS in brain. Primary microglial cells were incubated with fluorescent beads (green) for 2 hrs then incubated at 37˚C with medium then fixed and stained with DAPI (blue). Primary cells treated with 100ng/ml LPS. A) Images display the phagocytic cells in primary neonatal and adult brain rat microglia. B) Histogram of flourescence intensity /number of total cells. The graphs represent means±SEM obtained from three independent experiments (4 slides for each group and quantify 4 fields from each slide). Data were analysed using student's t-test. *, **, *** = $P<0.05$, $P<0.01$ and $P<0.001$ respectively.

A)



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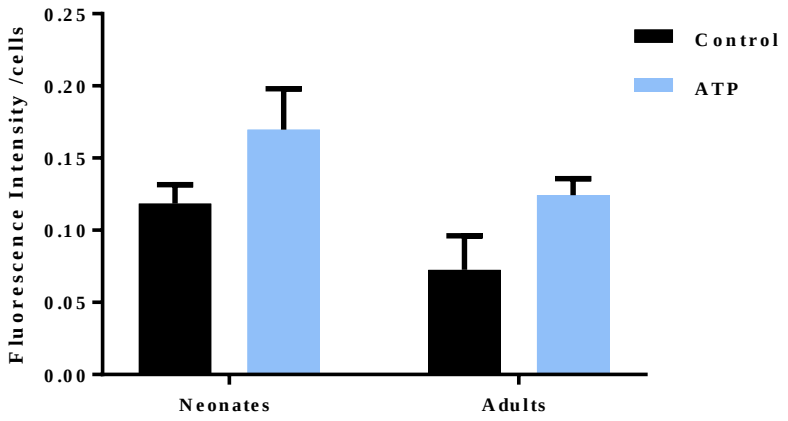
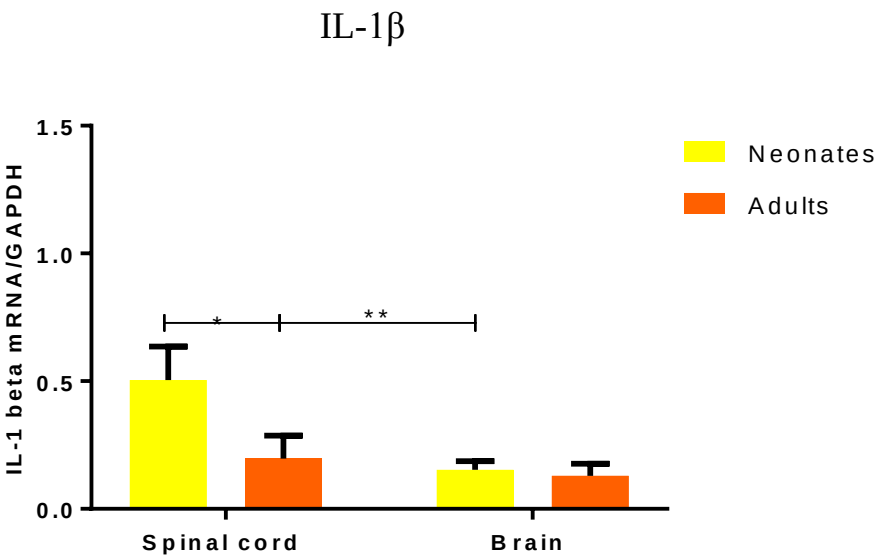


Figure 4.10. Microglia phagocytic activity impairment with age in response to ATP in brain. Primary microglial cells were incubated with fluorescent beads (green) for 2 hrs then incubated at 37˚C with medium then fixed and stained with DAPI (blue). Primary cells treated with 100µM ATP. A) Images display the phagocytic cells in primary neonatal and adult brain rat microglia. B) Histogram of fluorescence intensity/number of total cells. The graphs represent means±SEM obtained from three independent experiments (4 slides for each group and quantify 4 fields from each slide). Data were analysed using student's t-test.

4.2.5 Age- and location-related basal cytokines levels in microglia

The exposure to systemic factors in spinal cord and brain may result in enhanced the inflammatory state of microglia through increased cytokines signalling. IL-1β is thought to play a predominant role in development of several age-related degenerative diseases, including AD (Heneka et al., 2013). In addition, Youm et al. (2013) has identified Nlrp3 inflammasome-mediated production of IL-1β as a critical link between innate immune activation and functional decline in the aging CNS. IL-1β expression was greater significantly in the spinal cord compared to brain in early life (spinal: 0.50±0.13, brain: 0.15±0.03; $P<0.05$). In addition, the mRNA expression levels of IL-1β was significantly higher in neonatal microglia compared to adult cells in spinal cord (neonates: 0.50±0.13, adult: 0.20±0.09; $P<0.01$), but not in brain (Figure 4.11A). However, the expression level of anti-inflammatory cytokine IL-10 was significantly higher in the spinal cord compared to brain in both neonatal and adult microglia (neonates (spinal: 0.98±0.23, brain: 0.18±0.09; $P<0.05$), adult (spinal: 0.75±0.16, brain: 0.15±0.06; $P<0.05$). IL-10 acts to inhibit pro-inflammatory mediator release, as well as reducing the recruitment of immune related glia cells in the CNS (Milligan and Watkins, 2009). The basal tissue expression of IL-10 was significantly decreased in adult microglia compared to neonatal cells in both spinal cord and brain (spinal (neonates: 0.98±0.23, adult: 0.75±0.16), $P<0.01$), brain (neonates: 0.18±0.09, adult: 0.15±0.06); $P<0.01$) (Figure 4.11B).

A)



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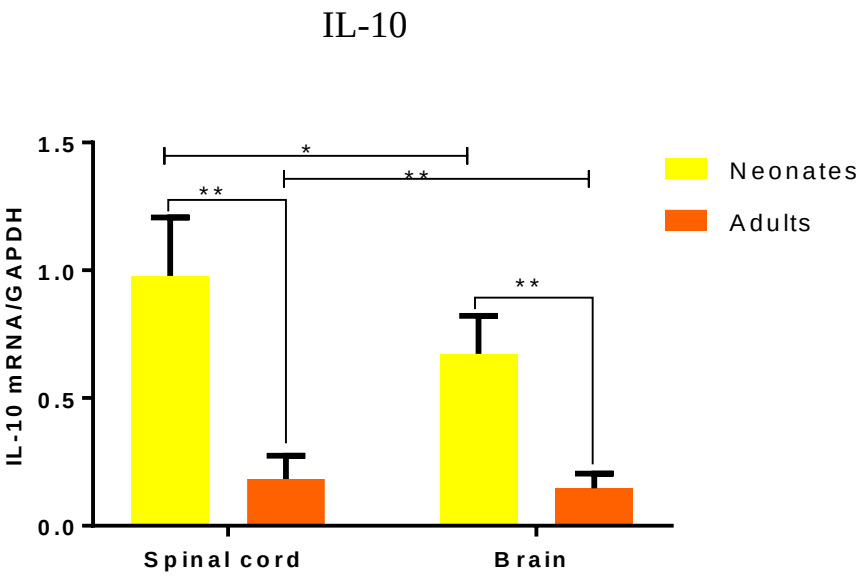
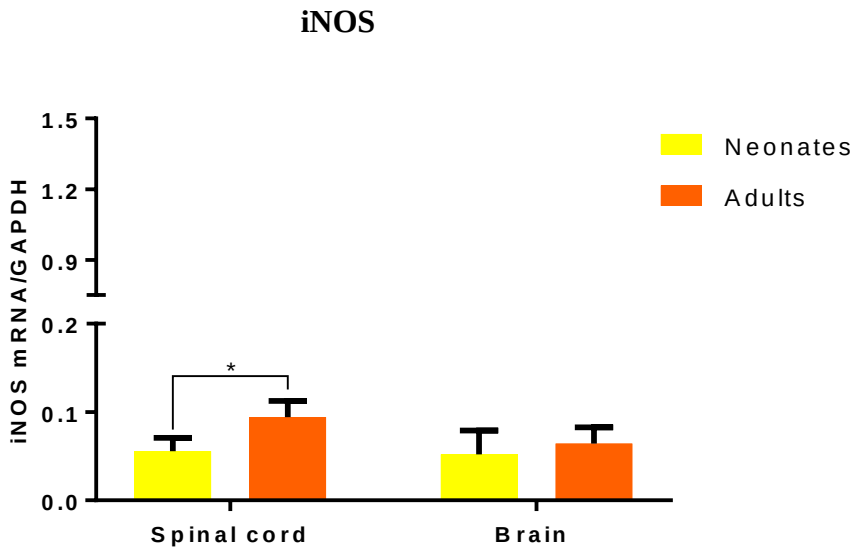


Figure 4.11. mRNA expression in neonatal and adult microglia in brain and spinal cord. Primary brain and spinal microglia were isolated from rat pups and adults and their basal inflammatory cytokines expression IL-1β (A) and IL-10 (B) was measured using Taqman PCR and normalized to GAPDH expression. The graphs represent means±SEM obtained from three independent experiments. Data were analysed by student's t-test. *, ** = P<0.05 and P<0.01 respectively.

4.2.6 Age- and location-related iNOS mRNA expression and NO production in microglia

Expression level of iNOS was higher in the spinal microglia compared to brain in both neonates and adult, but not statistically significant (neonates (spinal: 0.06±0.01 vs brain: 0.05±0.03; P=0.8106), (adult (spinal: 0.09±0.02 vs brain: 0.06±0.02; P=0.2798) (Figure 4.12A). In addition, the mRNA expression levels of iNOS was significantly lower in neonatal microglia compared to adult cells in spinal cord (neonates: 0.04±0.01 vs adult: 0.09± 0.02; P<0.05), and not significant in brain microglial cells (neonates: 0.05±0.03 vs adult: 0.06± 0.02; P=0.7219) (Figure 4.12A). Inconsistent with the iNOS mRNA expression levels, the NO production was significantly higher in the brain compared to spinal cord in both neonatal (spinal: 1.18±0.06 vs brain: 1.70±0.12; P<0.05) and adult microglia (spinal: 1.78±0.06 vs brain: 2.21±0.19; P<0.05). Moreover, the basal tissue level of NO was significantly increased in adult microglia compared to neonatal cells in both spinal cord (neonates: 1.18±0.06 vs adult: 1.78±0.06; P<0.01) and brain (neonates: 1.70±0.12 vs adult: 2.21±0.19; P<.05) (Figure 4.12B).

(A)



(B)

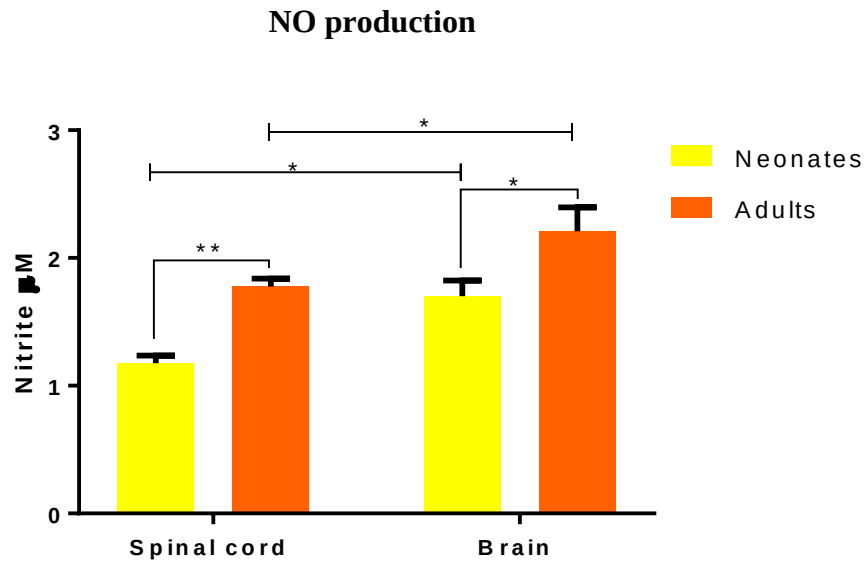


Figure 4.12. Tissue-level iNOS mRNA expression and NO production in neonatal and adult microglia in brain and spinal cord. Primary brain and spinal microglia were isolated from rat pups and adults and their basal iNOS expression (A) and NO production (B) were measured using Taqman qPCR and Griess reagent respectively. The graphs represent means±SEM obtained from three independent experiments. Data were analysed by student's t-test. *, ** = P<0.05 and P<0.01 respectively.

4.2.7 Differential changes in inflammatory cytokine expression in response to PAMPs and DAMPs in adult and neonatal spinal and brain microglia

4.2.7.1 Effect of PAMPs and DAMPs on IL-1β and IL-10 expressions in spinal microglia

IL-1β gene expression levels in the neonate and adult spinal cord microglia stimulated with PAMPs (LPS) and DAMPs (ATP and HMGB1) were measured using Taqman qPCR. Microglia stimulated with LPS showed a significant increase in IL-1β mRNA levels in both neonatal and adult spinal cells (198.60±14.73 and 124.7±18.58 fold respectively) (Figure 4.13A). ATP and HMGB1-stimulated adult spinal microglia displayed significant increase in IL-1β mRNA levels (15.89±0.89 and 10.17±1.26 respectively) (Figure 4.13B and C). In contrast, IL-1β mRNA expression levels in neonatal spinal microglia showed a much reduced response (1.64±0.47 and 4.09±1.26) upon stimulation with ATP and HMGB1 respectively (Figure 4.13B and C).

IL-10 mRNA was increased to a greater extent in neonatal microglia (16.79±1.29 fold) relative to adult cells (8.15±1.63 fold) after stimulation with LPS (Figure 4.13A). IL-10 mRNA also significantly increased in neonatal spinal microglia, after stimulation with ATP and HMGB1 (3.78±0.65 and 4.54±0.43 fold respectively) (Figure 4.13B and C), compared to adult cells IL-10 mRNA decreased slightly after ATP exposure, and showed a modest increase when stimulated the cells with HMGB1 (Figure 4.13B and C). Taken together, these findings suggest that adult microglia showed pro-inflammatory response following stimulation with PAMPs and DAMPs. However, the PAMPs and DAMPs stimulations trigger an anti-inflammatory response in neonatal microglia.

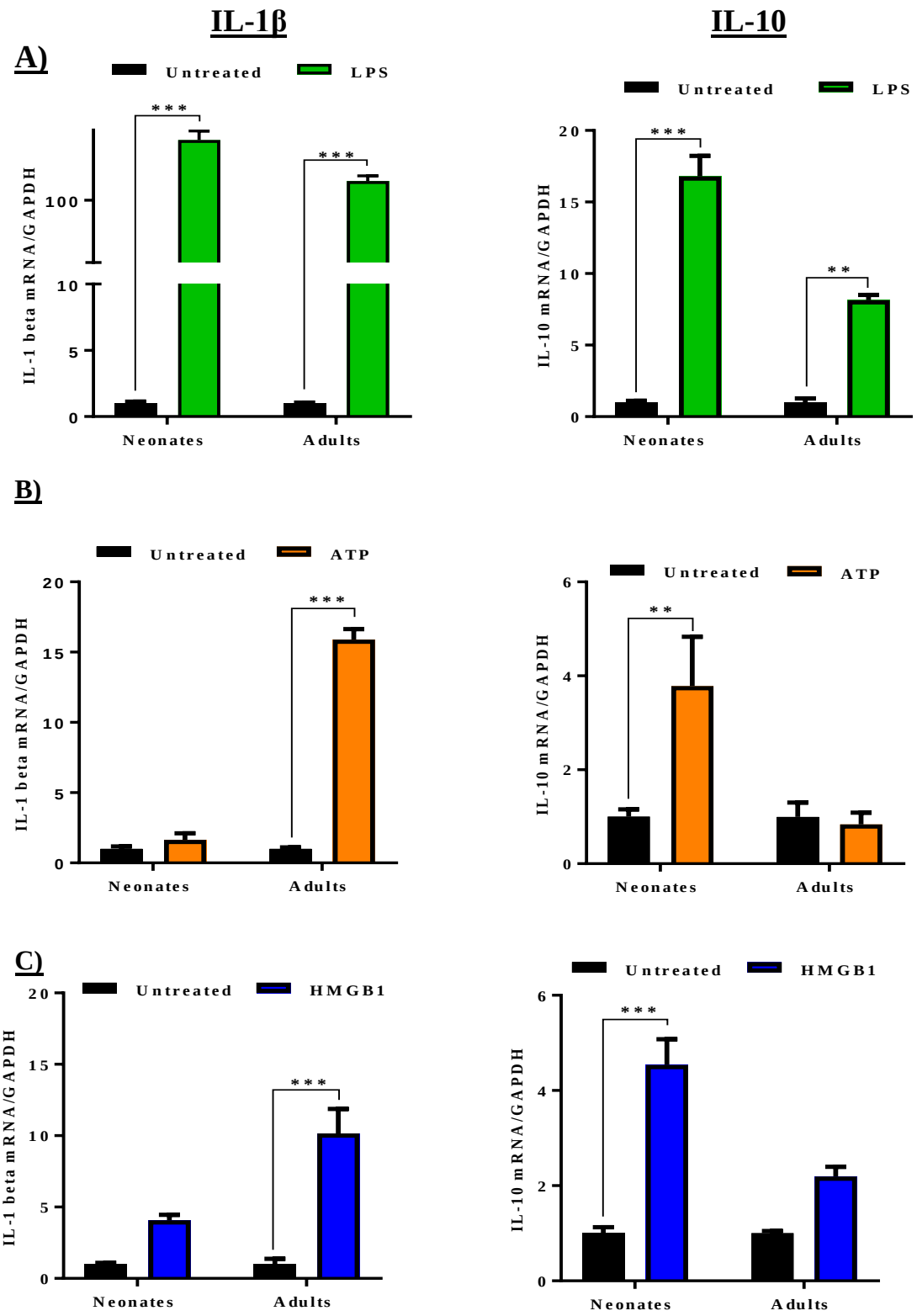


Figure 4.13. The mRNA expression of IL-1β and IL-10 in spinal microglia in response to stimulation with PAMPs and DAMPs are significantly different in early life and adulthood. Primary spinal microglia were isolated from rat pups and adults and their response to stimulation with LPS (A), ATP (B) and HMGB1 (C) measured using Taqman qPCR to measure changes in the expression of IL-1β and IL-10, and normalized to GAPDH expression. The graphs represent means±SEM obtained from three independent experiments. Data were analysed by student's t-test. *** = P<0.001.

4.2.7.2 Effect of PAMPs and DAMPs on pro-inflammatory cytokine IL-1β and IL-10 in brain microglia

Brain microglia stimulated with LPS showed a significant increase in IL-1β mRNA in both neonatal and adult cells (66.47±4.38 and 21.89±5.12 fold respectively) (Figure 4.14A). ATP and HMGB1-stimulated adult brain microglia displayed a significant increase in IL-1β mRNA 7.94±0.83 and 8.96±1.66 fold respectively) (Figure 4.14B and C). However, IL-1β mRNA expression in neonatal spinal microglia showed a much reduced response (1.32±0.67 and 3.45±1.93 fold) upon stimulation with ATP and HMGB1 respectively (Figure 4.14B and C).

IL-10 mRNA transcript levels were significantly increased in neonatal brain microglia (16.60±1.44 fold) and in adult cells (11.98±1.76 fold) after stimulation with LPS (Figure 4.14A). IL-10 mRNA levels significantly increased in neonatal brain microglia, after stimulation with ATP and HMGB1 (3.36±0.49 and 4.87±0.52 fold respectively) (Figure 4.14B and C), compared to adult cells in which levels of IL-10 were largely unchanged (1.06±0.48) after ATP exposure, and increased modestly with exposure to HMGB1 (2.39±0.54 fold) (Figure 4.14B and C).

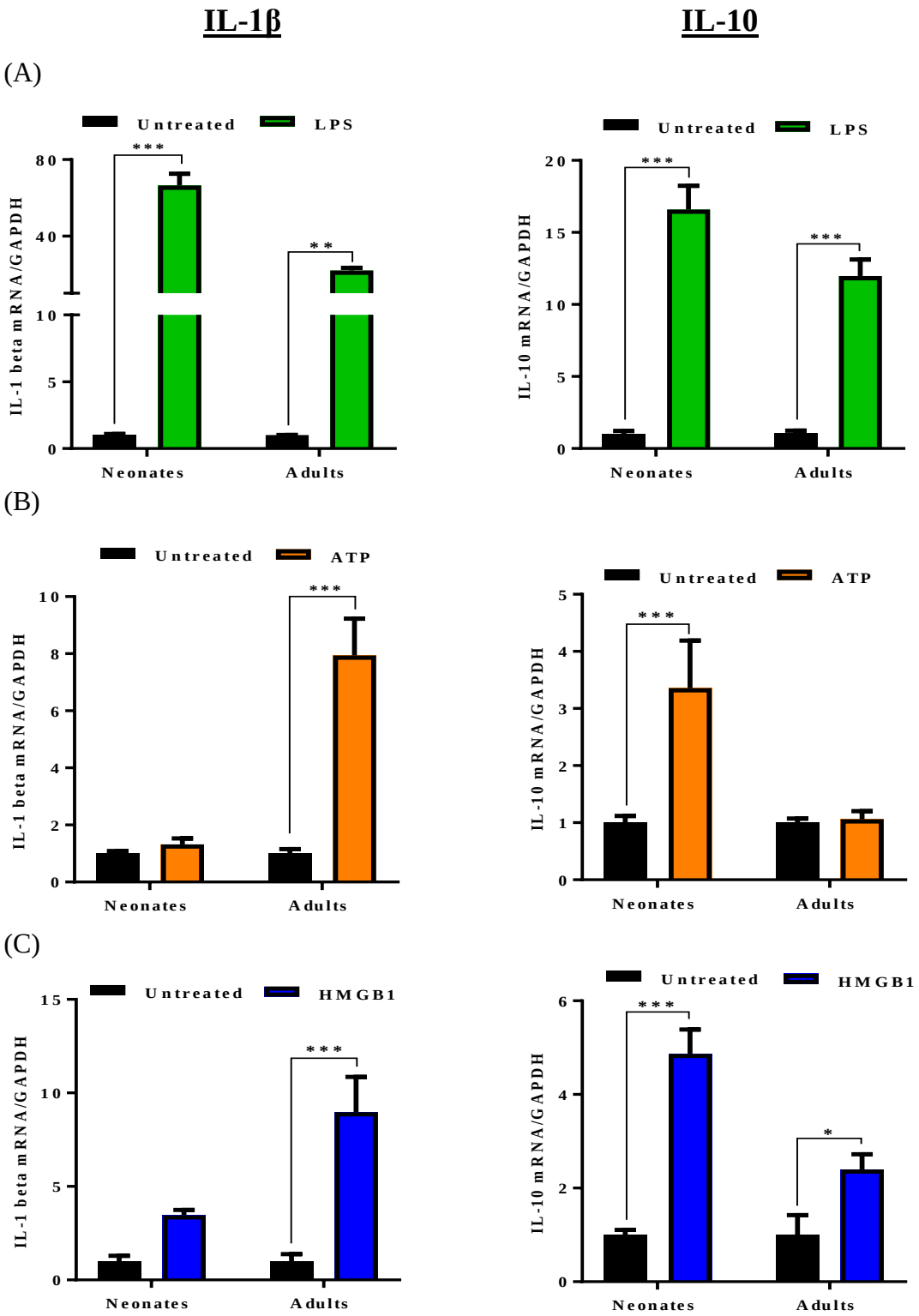


Figure 4.14. The mRNA expression of IL-1β and IL-10 in Brain microglia in response to stimulation with PAMPs and DAMPs are significantly different in early life and adulthood. Primary brain microglia were isolated from rat pups and adults and their response to stimulation with LPS (A), ATP (B) and HMGB1 (C) measured using Taqman PCR to measure changes in the expression of IL-1β and IL-10, and normalized to GAPDH expression. The graphs represent means±SEM obtained from three independent experiments. Data were analysed by student's t-test. **, *** = P<0.01 and P<0.001 respectively.

4.2.8 Differential changes in iNOS expression and NO production in response to PAMPs and DAMPs in microglia

4.2.8.1 Effect of PAMPs and DAMPs on the expression of iNOS and NO production in spinal microglia

qPCR analysis showed that iNOS mRNA transcript levels were highly significantly increased by LPS stimulation in both neonatal and adult microglia (427.5±4.05 and 124.60±5.42 respectively), and this was associated with a much smaller, but significant increase in nitrite concentration in the culture media (neonate: 2.62µM±0.07 vs adult: 2.24µM±0.08) (Figure 4.15A). ATP and HMGB1-stimulated neonatal and adult spinal microglia displayed increased iNOS mRNA expression levels (ATP: 2.36±0.38 vs 30.18±4.89; HMGB1: 7.88±2.17 vs 12.56±2.17 (neonates vs adult)) and there was an accumulation of nitrite in the culture media observed following exposure to ATP and HMGB1 (ATP: 4.37±0.27 vs 4.45±0.44, HMGB1: 2.79±0.14 vs 2.25±0.05, (neonates vs adult)) (Figure 4.15B and C).

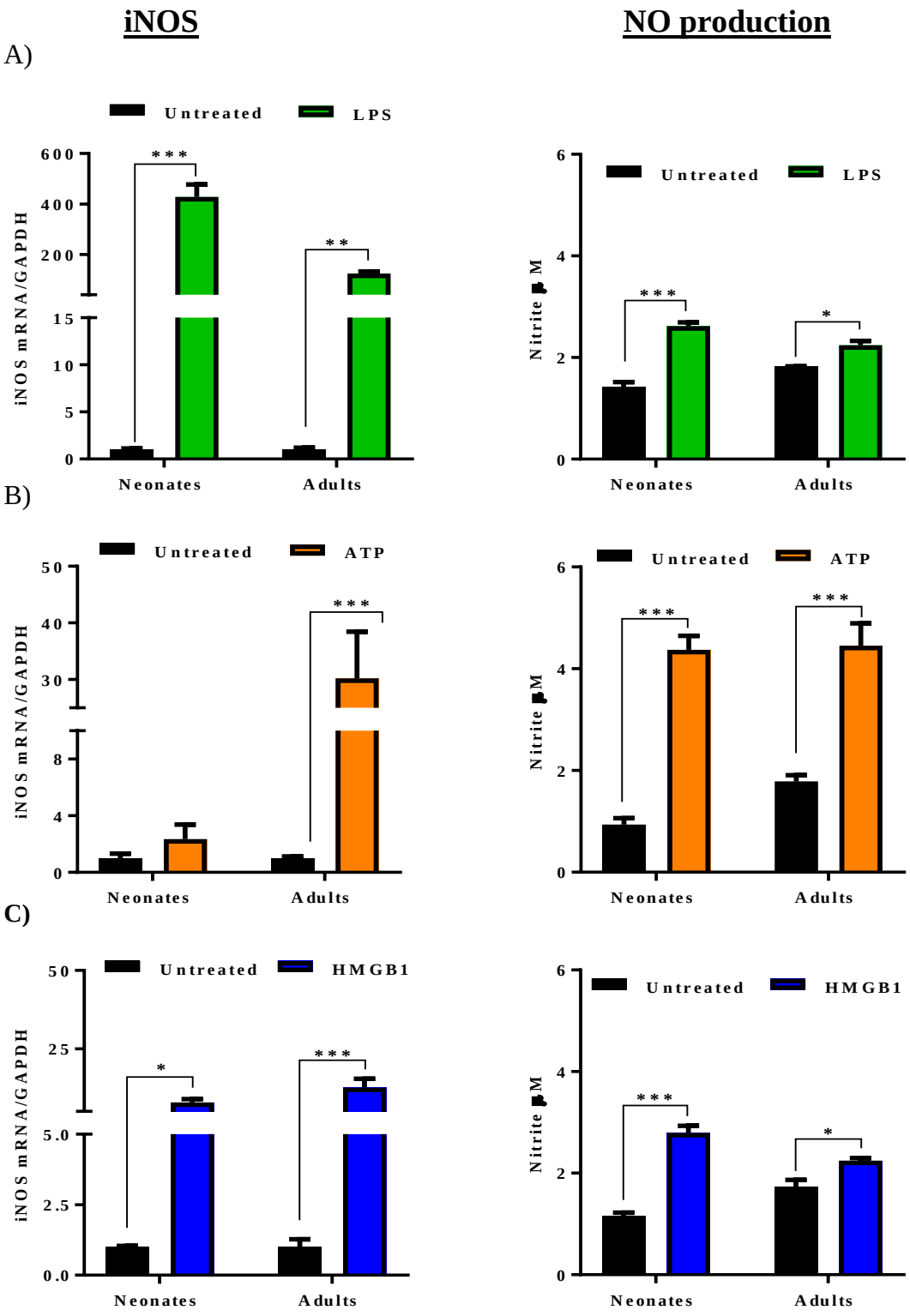


Figure 4.15. Changes of iNOS expression and nitrite accumulation upon LPS, ATP and HMGB1stimulation in neonatal and adult spinal microglia. Primary spinal microglia were isolated from rat pups and adults and their response to stimulation with LPS (A), ATP (B) and HMGB1 (C) measured using Taqman PCR to measure changes in the expression of iNOS and normalized to GAPDH expression. NO production was assessed by quantification of nitrite using the Griess reagent. The graphs represent means±SEM obtained from three independent experiments. Data were analysed by student's t-test. *, **, *** = P<0.05, P<0.01 and P<0.001 respectively.

4.2.8.2 Effect of PAMPs and DAMPs on the expression of iNOS and nitric oxide production in brain microglia

iNOS mRNA levels were significantly increased by LPS stimulation in both neonatal and adult brain microglia (433.10±14.73 and 49.32±4.11 respectively), and this was related to an increase in nitrite concentration in the culture media (neonate: 2.66µM±0.09 vs adult: 2.37µM±0.15) (Figure 4.16A). ATP and HMGB1-stimulated neonatal and adult brain microglia displayed increases in iNOS mRNA levels (ATP: 3.02±0.62 vs 7.47±1.10; HMGB1: 8.62±0.87 vs 30.26±2.98 (neonates vs adult)) and there was an accumulation of nitrite in the culture media observed following exposure to ATP and HMGB1 (ATP: 3.27±0.29µM vs 7.09±0.08µM, HMGB1: 2.92±0.08µM vs 2.82±0.13µM, (neonates vs adult)) (Figure 4.16B and C).

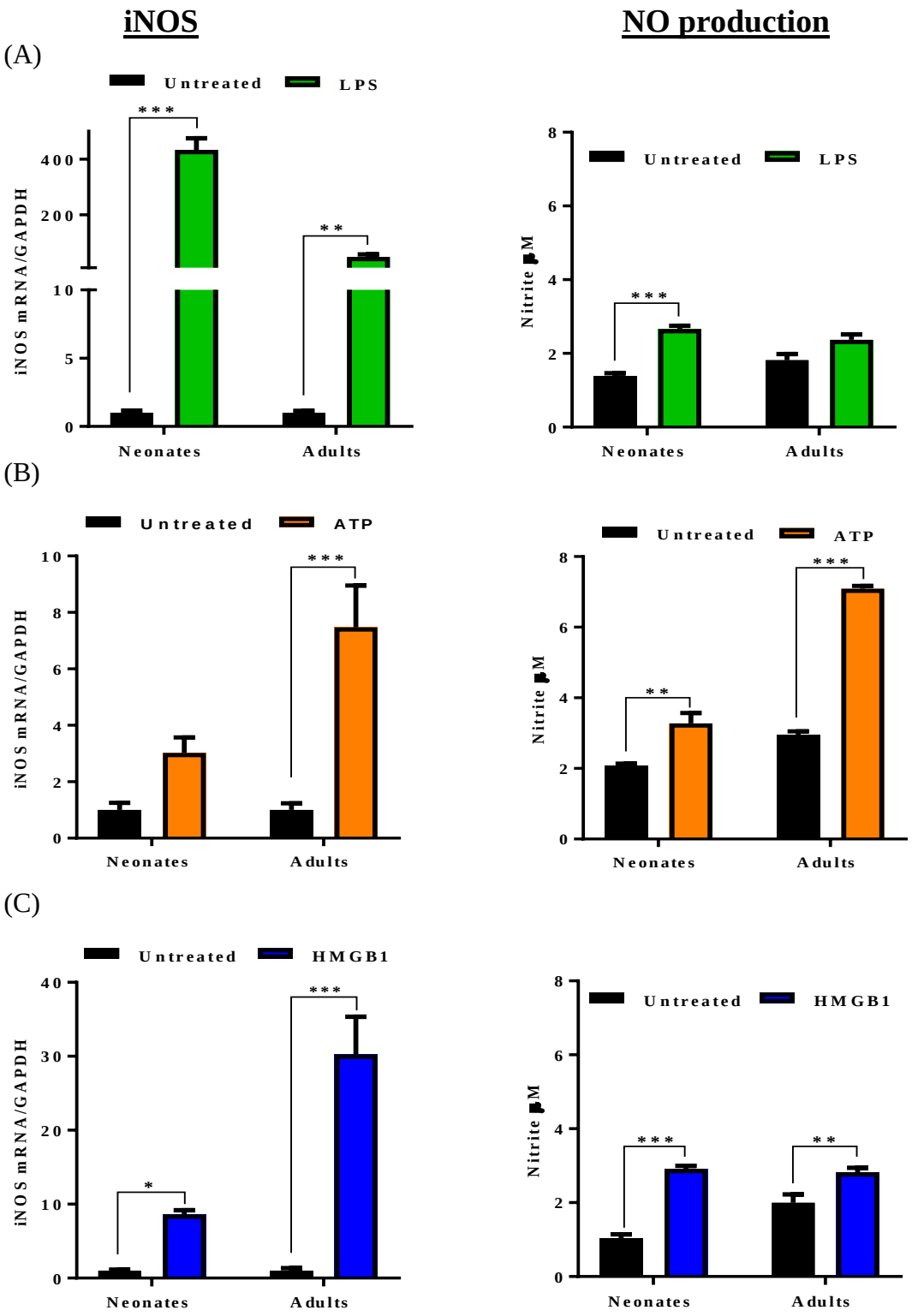


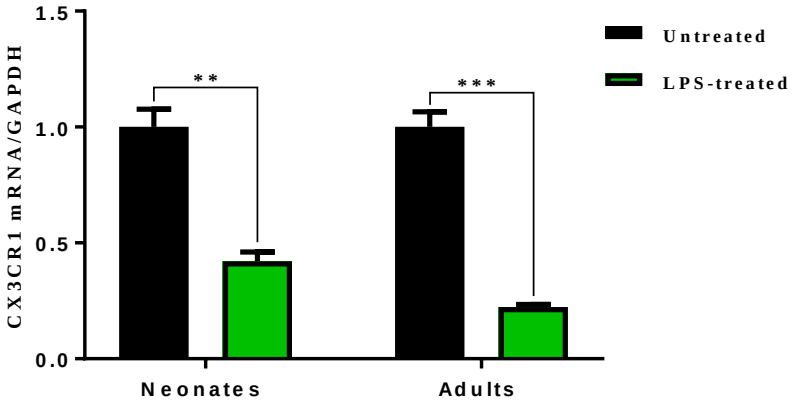
Figure 4.16. Changes of iNOS expression and nitrite accumulation upon LPS, ATP and HMGB1stimulation in neonatal and adult brain microglia. Primary brain microglia were isolated from rat pups and adults and their response to stimulation with LPS (A), ATP (B) and HMGB1 (C) measured using Taqman PCR to measure changes in the expression of iNOS and normalized to GAPDH expression. NO production was assessed by quantification of nitrite using the Griess reagent. The graphs represent means±SEM obtained from three independent experiments. Data were analysed by student's t-test. *, **, *** = P<0.05, P<0.01 and P<0.001 respectively.

4.2.9 Effect of PAMPs and DAMPs on CX3CR1 receptor in spinal and brain microglia

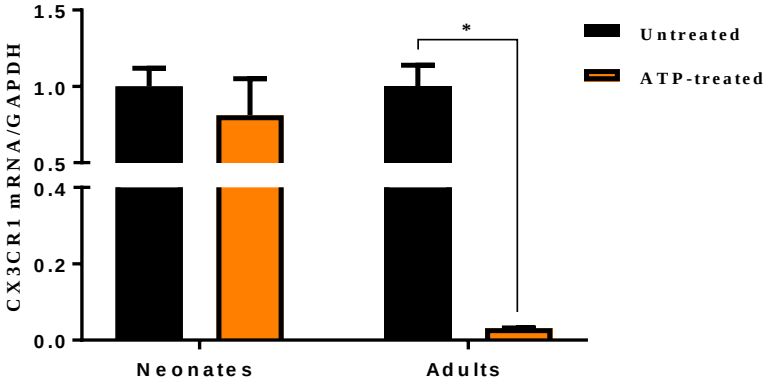
Fractalkine is primarily produced in neurons and a considerable body of evidence points to the importance of the interaction between neuronal fractalkine production and microglial activation state and function. Taqman qPCR was used to determine the expression of the Fractalkine receptor (CX3CR1) mRNA in primary cultures of rat spinal and brain microglia after exposure to PAMPs and DAMPs. CX3CR1 receptor mRNA was expressed in both neonatal and adult spinal microglia, however, exposure to LPS significantly reduced expression of fractalkine receptor mRNA in spinal microglia (25.0±0.003 vs 4.54±0.07 fold, neonate vs adult) (Figure 4.17A).

Interestingly, in neonatal spinal microglia, CX3CR1 mRNA levels did not change after challenge with ATP, whereas CX3CR1 mRNA levels significantly decreased 33.3±0.001 fold following ATP exposure in adult spinal microglia (Figure 4.17B). The CX3CR1 mRNA levels in both neonatal adult spinal microglia significantly decreased after HMGB1 stimulation (3.70±0.02 vs 2.00±0.16 fold, neonate vs adult) (Figure 4.17C).

(A)



(B)



(C)

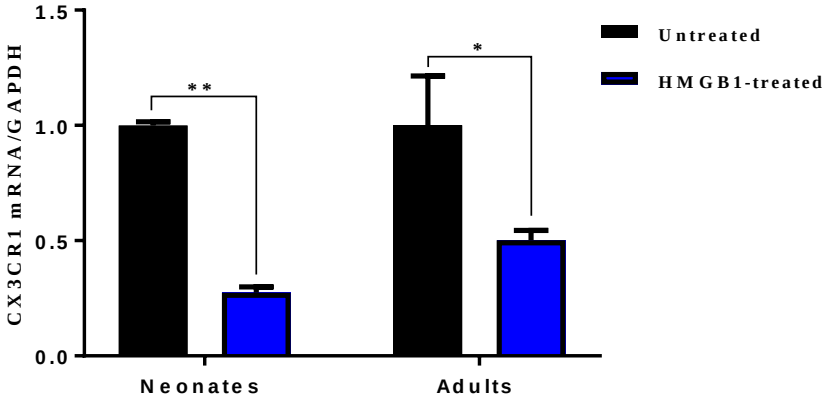
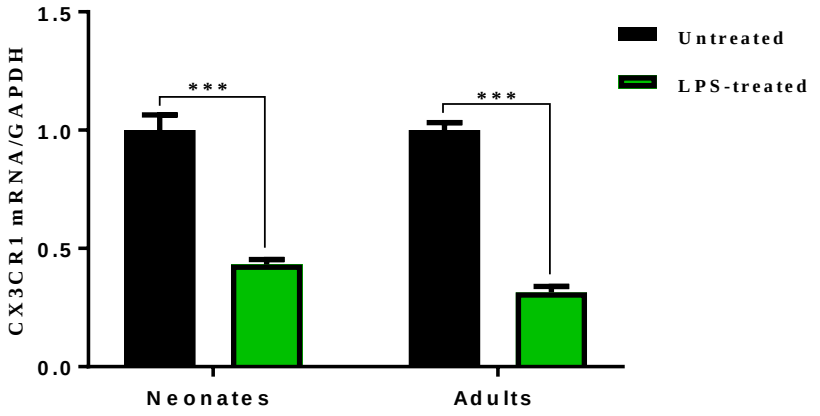


Figure 4.17. The mRNA expression of CX3CR1 in spinal microglia in response to stimulation with PAMPs and DAMPs in early life and adulthood. Primary spinal microglia were isolated from rat pups and adults and their response to stimulation with LPS (A), ATP (B) and HMGB1 (C) measured using Taqman PCR to measure changes in the expression of CX3CR1 and normalized to GAPDH expression. The graphs represent means±SEM obtained from three independent experiments. Data were analysed by student's t-test. *, **, *** = P<0.05, P<0.01 and P<0.001 respectively.

CX3CR1 receptor mRNA was clearly expressed in both neonatal and adult brain microglia, mRNA expression levels of fractalkine receptor in microglia were suppressed significantly after LPS stimulation (0.43±0.06 vs 0.31±0.07, neonate vs adult) (Figure 4.18A).

Interestingly, mRNA expression levels of CX3CR1 in neonatal and adult brain microglia did not change significantly after stimulation with ATP (Figure 4.18B). The CX3CR1 mRNA transcript levels in neonatal and adult brain microglia significantly decreased 0.18±.02 and 0.43±0.20 after HMGB1 stimulation (Figure 4.18C).

(A)



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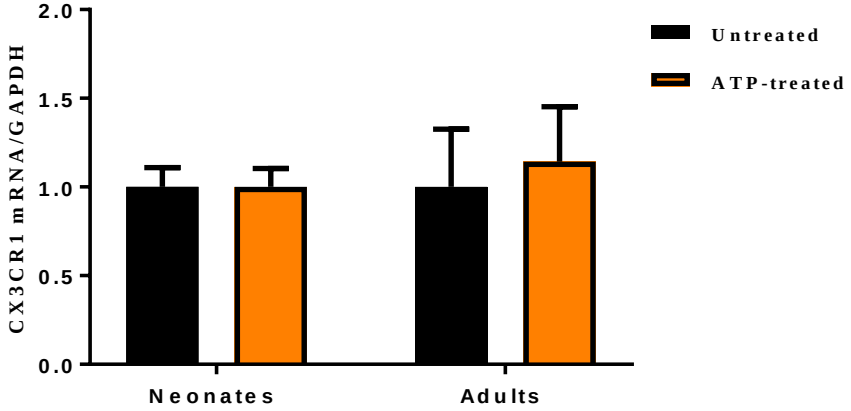

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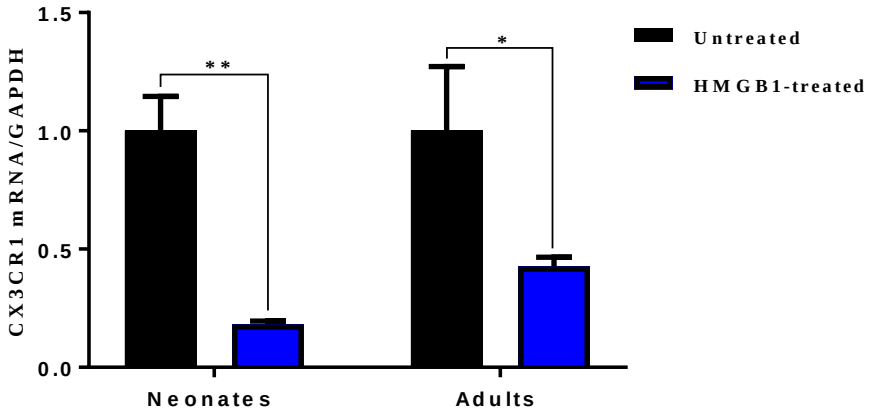


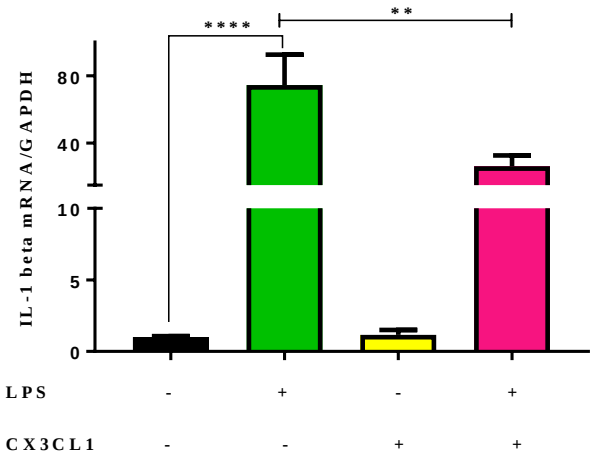
Figure 4.18. The mRNA expression of CX3CR1 in brain microglia in response to stimulation with PAMPs and DAMPs in early life and adulthood. Primary brain microglia were isolated from rat pups and adults and their response to stimulation with LPS (A), ATP (B) and HMGB1 (C) measured using Taqman PCR to measure changes in the expression of CX3CR1 and normalized to GAPDH expression. The graphs represent means±SEM obtained from three independent experiments. Data were analysed by student's t-test. *, **, *** = $P<0.05$, $P<0.01$ and $P<0.001$ respectively.

4.2.10 The effects of fractalkine following microglial exposure to LPS

4.2.10.1 The role of fractalkine in modulating the phenotype of the LPS-activated neonatal and adult spinal microglial cells

In the presence of CX3CL1, LPS-stimulated neonatal microglia expressed lower mRNA levels of IL-1β compared to LPS stimulation alone (LPS 74.58±1.81 fold; LPS+CX3CL1: 26.25±0.63 fold, P<0.001) (Figure 4.19A). Interestingly, CX3CL1 also increased IL-10 mRNA in LPS-activated neonatal microglia (LPS 8.05±1.42 fold; LPS+CX3CL1: 21.08±2.76 fold, P<0.001) (Figure 4.19B).

(A)



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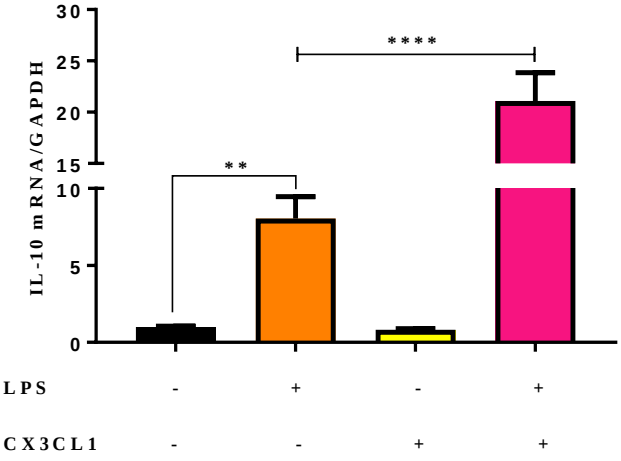
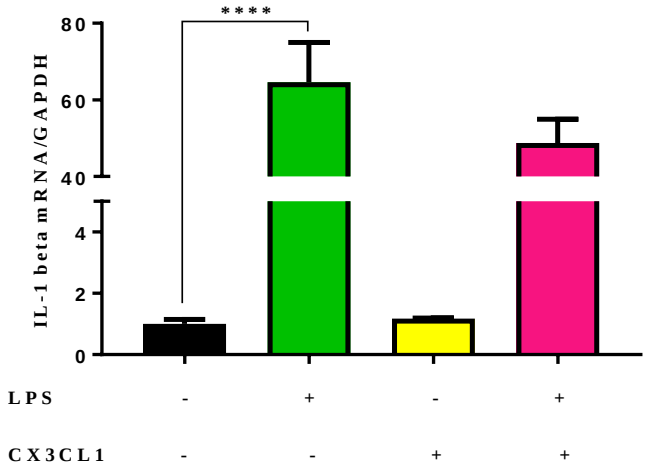


Figure 4.21. Fractalkine modulates the expression of inflammatory genes in neonatal spinal microglia upon LPS activation. Microglia were treated with fractalkine 100nM for 24 hours then activated with 100ng/ml LPS as described in Materials and Methods chapter. (A) The gene expression of IL-1β in neonatal microglia, (B) The gene expression of IL-10 in neonatal microglia. The gene expression was measured by Taqman qPCR and normalized to GAPDH expression. The graphs represent means±SEM obtained from three independent experiments. Data were analysed by one-way ANOVA with Bonferroni post-tests. *, **, *** = P<0.05, P<0.01 and P<0.001 respectively.

In contrast to neonatal microglia, Fractalkine was not able to significantly alter the mRNA levels of IL-1β in LPS-activated adult microglial cells (LPS 64.60±1.55; LPS+CX3CL1: 48.77±1.08) (Figure 4.20A). As shown in Figure 4.20B, fractalkine did not affect the mRNA expression levels of anti-inflammatory cytokine IL-10 (LPS 7.21±0.97; LPS+CX3CL1: 8.36±1.34). All together these findings reveal that CX3CL1 can induce functional changes on activated microglia and it is able to supress LPS-stimulated microglia.

(A)



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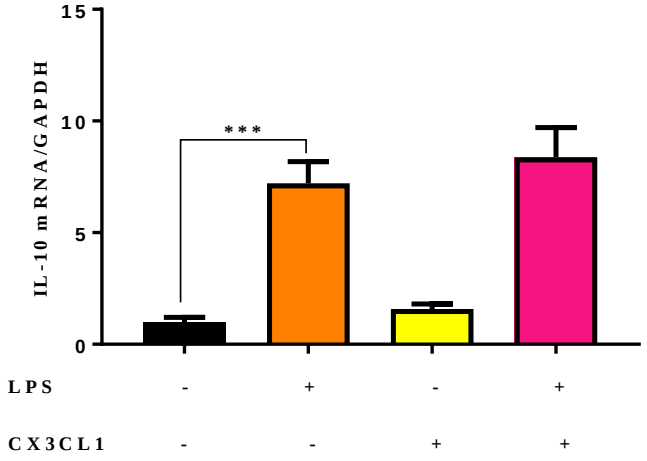
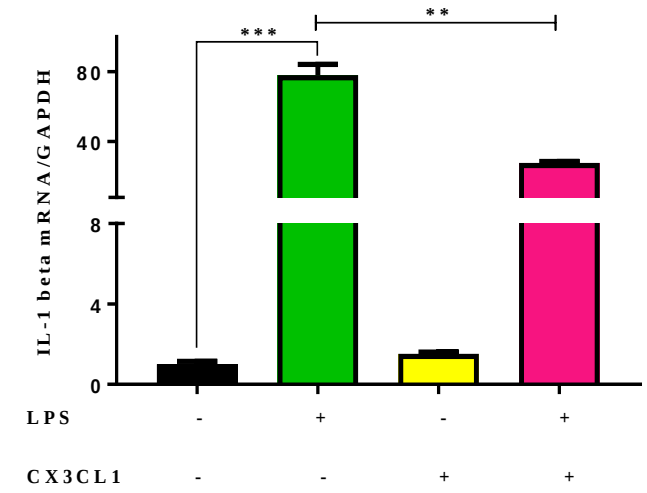


Figure 4.22. Fractalkine modulates the expression of inflammatory genes in spinal adult microglia upon LPS activation. Microglia were treated with fractalkine 100nM for 24 hours then activated with 100ng/ml LPS as described in Materials and Methods chapter. (A) The gene expression of IL-1β in neonatal microglia, (B) The gene expression of IL-10 in neonatal microglia. The gene expression was measured by Taqman qPCR and normalized to GAPDH expression. The graphs represent means±SEM obtained from three independent experiments. Data were analysed by one-way ANOVA with Bonferroni post-tests. *, **, *** = $P<0.05$, $P<0.01$ and $P<0.001$ respectively.

4.2.10.2 The role of fractalkine in modulating the phenotype of the LPS-activated neonatal and adult brain microglial cells

In the presence of CX3CL1, LPS-stimulated neonatal brain microglia expressed lower mRNA levels of IL-1β compared to LPS stimulation alone (LPS 78.06±1.34; LPS+CX3CL1: 27.84±0.82, P<0.01) (Figure 4.21A). CX3CL1 also increased IL-10 mRNA in LPS-activated neonatal microglia (LPS 12.02±0.58; LPS+CX3CL1: 34.39±2.76, P<0.001) (Figure 4.21B).

(A)



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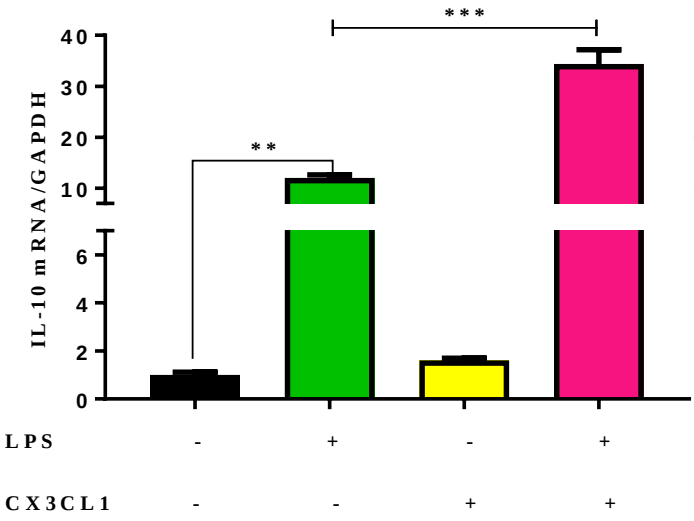
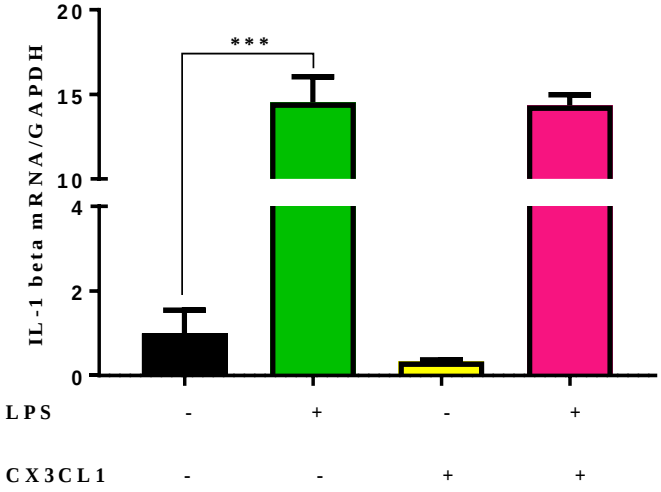


Figure 4.23. Fractalkine modulates the expression of inflammatory genes in neonatal brain microglia upon LPS activation. Microglia were treated with 100nM for 24 hours then activated with 100ng/ml LPS as described in Materials and Methods chapter. (A) The gene expression of IL-1β in neonatal microglia, (B) The gene expression of IL-10 in neonatal microglia. The gene expression was measured by Taqman qPCR and normalized to GAPDH expression. The graphs represent means±SEM obtained from three independent experiments. Data were analysed by one-way ANOVA with Bonferroni post-tests. *, **, *** = P<0.05, P<0.01 and P<0.001 respectively.

To investigate that fractalkine could modulate the inflammatory response of LPS-induced adult brain microglia, mRNA expression levels of IL-1β and IL-10 were measured using Taqman qPCR. Fractalkine was not able to change significantly the mRNA levels of IL-1β in LPS-stimulated adult microglial cells (LPS 14.53±1.52; LPS+CX3CL1: 14.35±0.64) (Figure 4.22A). As indicated in Figure 4.22B, CX3CL1 did not showed any significant effect on the mRNA expression levels of anti-inflammatory cytokine IL-10 (LPS 10.65±1.40; LPS+CX3CL1: 11.98±1.18).

A)



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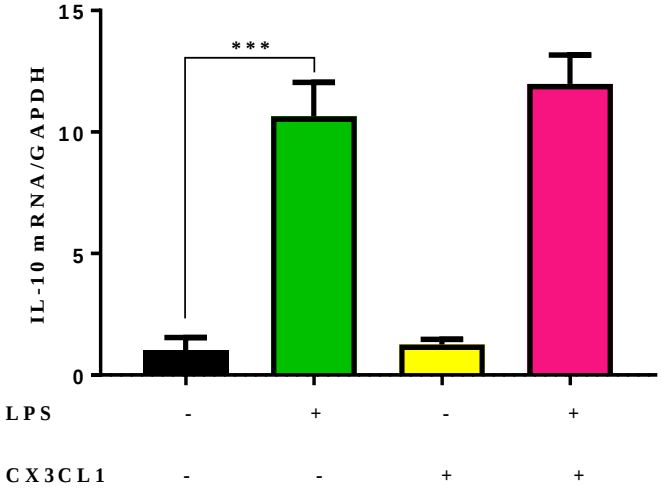


Figure 4.24. Fractalkine modulates the expression of inflammatory genes in brain microglia upon LPS activation. Microglia were treated with 100nM for 24 hours then activated with 100ng/ml LPS as described in Materials and Methods chapter. (A) The gene expression of IL-1β in neonatal microglia, (B) The gene expression of IL-10 in neonatal microglia. The gene expression was measured by Taqman qPCR and normalized to GAPDH expression. The graphs represent means±SEM obtained from three independent experiments. Data were analysed by one-way ANOVA with Bonferroni post-tests. *, **, *** = P<0.05, P<0.01 and P<0.001 respectively.

4.3 Discussion

Novel data presented here provides significant insights into microglia functional changes during development. The work shows that inflammatory cytokines differ in their expression profile in neonatal and adult microglia in brain and spinal cord. In addition, PAMP and DAMP exposures produced different changes in gene expression in neonatal and adult microglia. qPCR after ATP and HMGB1 challenges revealed neonatal microglia profile with increased expression of anti-inflammatory gene IL-10. Furthermore, adult microglia failed to increase IL-10 expression after DAMP challenges, however adult cells did show increased IL-1β expression and showed less phagocytic activity compared to neonatal cells. These observations suggest that microglial function is affected by the microenvironment, brain or spinal cord, the stage of development and the way in which they are stimulated.

Most studies of microglia have been performed using neonatal primary microglia isolated from the cerebral cortex as they are easier to isolate and culture than primary adult microglia. The current chapter present consistent and efficient procedures to isolate neonatal and adult single cell microglia from the whole rat brain as well as the whole rat spinal cord. qPCR assay for the cytokines suggest that no activation of microglial cells was observed after the isolation procedure. As a result, microglia reserved their ability to respond to immunogenic stimuli such as LPS and ATP, and the highly expressed IL-1β as a key inflammatory cytokine following stimulation. Moreover, the phagocytic activity of isolated microglia to remove fluorescence beads was shown in culture. Overall, the purification procedures revealed consistent high purities and maintain vital functional properties. Thus, the isolated microglia are likely

to sufficiently represent the real *in vivo* state and allow to study the phenotype and functions of microglial cells in the healthy and diseased brain or spinal cord.

In the current study, the basal phagocytic potential of unstimulated and LPS- and ATP-induced microglia was investigated, in order to understand the normal homeostatic changes that occur within the postnatal period. The findings of this study showed that the phagocytic activity decreased in adult microglia compared to neonatal cells in both unstimulated and stimulated microglia. In addition, These decreases in phagocytic activity of microglia have been involved in the pathogenesis of age-related diseases such as AD (Sokolowski and Mandell, 2011). Microglial phagocytic mechanisms may occur via signalling of the fractalkine to its receptor on microglia CX3CR1 (Hughes et al., 2002), and depletion of microglial CX3CR1 receptor in stimulated adult microglia may result in decreases of phagocytic activity of microglia. Microglia can phagocytise healthy cells, progenitor cells and apoptotic cells during CNS development (McArthur et al., 2010, Cunningham et al., 2013). Future research will need to determine what factors may be regulating microglial phagocytosis. Taken together, these age differences in microglial phagocytic activity may impact the development and life-long functions of the CNS in both steady state and in response to early life disturbances including inflammation and injury.

Microglia have the ability to detect deviations in the microenvironment through expression of functional range of immunological mediators that help them to react with environmental stimuli (Kettenmann et al., 2011). The data of this study show that microglia functions are greatly affected by microenvironment and age difference. At baseline levels, the inflammatory cytokines are expressed significantly through CNS

and their expression profile is deferent in brain and spinal cord. Remarkably, mRNA expression of IL-1β and IL-10 were higher in the spinal cord compared with the brain in both neonatal and adult microglia, see Figure 4.11. These findings are in line with a previous study (Ritzel et al., 2015) that reported the inflammatory cytokine levels were higher in spinal cord compared with brain in both young and aged animals. The higher basal cytokine levels in the spinal microglia may be associated with the high activated state in spinal cord, particularly with age. Despite these observations, age-related disorders of the brain are higher compared with those in the spinal cord, concluding that cytokines profile may not truly reflect physiological functions. A possible explanation for these regional differences may reveal changed blood-CNS barrier permeability and increased cytokine transport, leading to systemic factors exposure (Schnell et al., 1999, Bartanusz et al., 2011, Zhang and Gensel, 2014).

The microglia response is the first line of defence against pathogens in CNS (Takeuchi and Akira, 2010, Iwasaki and Medzhitov, 2015). As mentioned in the general introduction chapter, innate immune cells including microglia and astrocytes express a wide variety of PRRs which, following recognition of danger signals, initiate the production of immune mediators both pro- and anti-inflammatory cytokines (Iwasaki and Medzhitov, 2015). PRRs recognize PAMPs present in pathogens and DAMPs released by tissue damage (Takeuchi and Akira, 2010). As detection of PAMPs and DAMPs are crucial for the organism homeostasis, PAMPs recruits an immune response pointed at pathogen clearance, whereas stimulation of PRRs through DAMPs aims at the resolution of tissue damage (Lampron et al., 2013, Iwasaki and Medzhitov, 2015).

The elevated basal anti-inflammatory cytokine response to PAMP and DAMPs in neonatal microglia reported here suggests that the microglial response in neonates is skewed in an anti-inflammatory direction. In contrast to stimulated neonatal microglia, adult microglia stimulation triggers a pro-inflammatory response, characterised by a significant increase in IL-1β levels. Importantly, in adult, microglia from both brain and spinal cord show an obvious increase in cell size, NO production and cytokines secretion, in addition to decreases in phagocytic activity. There is previous evidence supporting the findings of this study, where microglial cells are very heterogeneous in the morphology and function. Scheffel et al. (2012), examined microglial heterogeneity in the postnatal maturation, and showed that microglia differentially respond to different stimulus, these responsive phenotypes variable with age and throughout CNS regions. Neonatal microglia display high proliferative capacity, an amoeboid morphology and phagocytic activity (Giulian and Baker, 1986), whereas adult microglia are implicated in the immune surveillance (Nimmerjahn et al., 2005), they are sensitive to pathologic changes that related to neurodegenerative disorders (Ponomarev et al., 2005).

Here, results showed that in neonatal microglial cells, ATP and HMGB1 were not able to increase the mRNA expression level of IL-1β, despite significant increase of these cytokine in response to LPS, ATP and HMGB1 in adult microglia, see Figure 4.7. This supports previous reports that microglial activation markers such as TNF-α are not increased in the early life of rat following sciatic nerve injury (SNI) (Moss et al., 2007, Costigan et al., 2009). Instead of a pro-inflammatory response, LPS, ATP and HMGB1 in neonates induced a significant increase in the expression of IL-10, see Figure 4.9. These findings are consistent with a previous study (Werry et al., 2011)

that reported LPS exposure to spinal microglia isolated from neonates significantly increased IL-10 mRNA expression *in vitro*. IL-10 inhibits pro-inflammatory mediator release and reduces the activation of immune cells in the CNS (Ponomarev et al., 2005, Milligan and Watkins, 2009). Norden et al. (2014) suggested a mechanism by which IL-10 reduces pro-inflammatory cytokine expressions in the CNS. They reported that IL-10 redirected astrocytes to control the activation of microglial cells in a TGF-β dependent pathway. consistent with this notion, astrocytes in the CNS have higher expression of IL-10R1 and they are influenced by anti-inflammatory effects of IL-10 more than other CNS cells (Lobo-Silva et al., 2016, Norden et al., 2016). In addition, astrocyte-microglia co-culture system is showed that the astrocytes facilitated the anti-inflammatory effects of IL-10 on microglia by reducing the expression of pro-inflammatory cytokines such as IL-1β and enhancing the expression of CX3CR1 and IL-4 receptor in primary microglia (Norden et al., 2014).

In terms of iNOS mRNA expression and NO production, changes in gene expression are supported by alterations in the activity of the encoded protein. Although some reports could not detect any NO production by adult microglial cells (Brannan and Roberts, 2004) or stated a similar or reduced microglial NO generation with age in response to LPS (Xie et al., 2003), the observations from this study showed that adult spinal and brain microglial cells significantly produce NO following LPS stimulations, and this finding is in line with (Moussaud and Draheim, 2010). The findings of this study showed that the DAMPs (ATP and HMGB1) are able to trigger significant NO production in both neonatal and adult microglial cells in spinal cord and brain. This is in line with previous studies that reported that ATP and HMGB1are able to generate NO in cultured microglia (Ohtani et al., 2000, Duan et al., 2009, Gao et al., 2011).

However, brain adult microglia significantly generate more NO than neonatal microglia.

Upon activation of microglia, the microglial protease cathepsin S cleaves CX3CL1 on the neuronal membrane surface, which acts on microglial CX3CR1 to mediate anti-inflammatory effects (Zhuang et al., 2007). Based on these findings, the ability of CX3CL1 to modulate the shift of microglia from a detrimental, neurotoxic phenotype controlled by the release of pro-inflammatory cytokines such as IL-1β to neuroprotective phenotype associated with the expression of anti-inflammatory cytokines such as IL-10, was assessed by experiments where CX3CL1 was added exogenously to LPS-induced neonatal and adult microglia. The findings of this study showed that CX3CL1 exerted anti-inflammatory effects in LPS-activated neonatal microglia by reducing the expression of IL-1β and inducing the expression of IL-10. This supporting its ability to supress the release of pro-inflammatory molecules typically occur after classical activation in response to pathogens or chronic CNS inflammation (Zhuang et al., 2007). In contrast, the fractalkine was not able to attenuate the LPS-activated adult microglia. One possible explanation for this observation that the CX3CR1 is reduced robustly after LPS chellenge (Figure 4.20A and 4.21A).

These data support previous *in vivo* investigations that indicate that neonatal nerve injury causes an "anti-inflammatory" immune response, characterised by significant increases in IL-10 in the spinal dorsal horn (McKelvey et al., 2015). In addition, McKelvey et al. (2015) demonstrated that blockade the anti-inflammatory activity with intrathecal functional anti-IL-10 triggers a hypersensitivity to mechanical

stimulation in infant nerve injured mice. In the current study, these age-related changes are reproducible in cultured microglia where IL-10 production is enhanced in neonatal microglia compared to adult, however, they also produce significant and comparable levels of IL-1β to adult cells. All together, these observations suggest that microglial activities and functions in the developing CNS may be different from those in the mature CNS, and it is in line with the notion that in the early postnatal period; the main role of microglia may be involved in building of CNS, while in adulthood, microglial function change to local surveillance manner (Nikodemova et al., 2015).

Observations from the current study showed differences in microglial cells response to PAMP and DAMPs in the same age. Microglial activation can occur through different signalling pathways. For instance, LPS activate microglia through TLR4 signalling pathway (Zhang et al., 2015), which results in activation of NF-$_k$B, interferon response factor (IRF3) and MAPK pathways (Wesche et al., 1997, Jiang et al., 2003). However, ATP triggers signalling in microglia by activating its specific receptors such as P2X4, P2X7 and P2Y12, which leads to the elevation of phosphatidylinositol 3,4,5-triphosphate (PIP3), which mediate subsequently an increase in intracellular calcium (Inoue et al., 1998, Irino et al., 2008) and outward potassium current (Swiatkowski et al., 2016). Moreover, the stimulation of P2X7 receptors causes an activation of MAP kinases and extracellular signal–regulated kinases (ERKs) (Hide et al., 2000, Lu et al., 2009). On the other hand, HMGB1 mediates cytokine release, inflammation and tissue injury via several receptors, including RAGE receptor, TLR-4, and TLR-2 (van Beijnum et al., 2008).

In summary, Microglia show distinct gene expression profiles and phagocytic activity in the early postnatal and adult periods, this is reflecting their specific functions during development. At adulthood, microglial cells appear to begin polarisation towards a pro-inflammatory phenotype which characterized by significant increases in IL-1β. However, PAMPs and DAMPs stimulations trigger an anti-inflammatory immune response in neonatal microglia with significant increases in IL-10. Age-related changes in gene expression profiles of microglia as revealed in this study may induce different microglial responses to physiological and pathological challenges, which in turn could significantly affect CNS susceptibility and the result of many CNS diseases at specific ages.

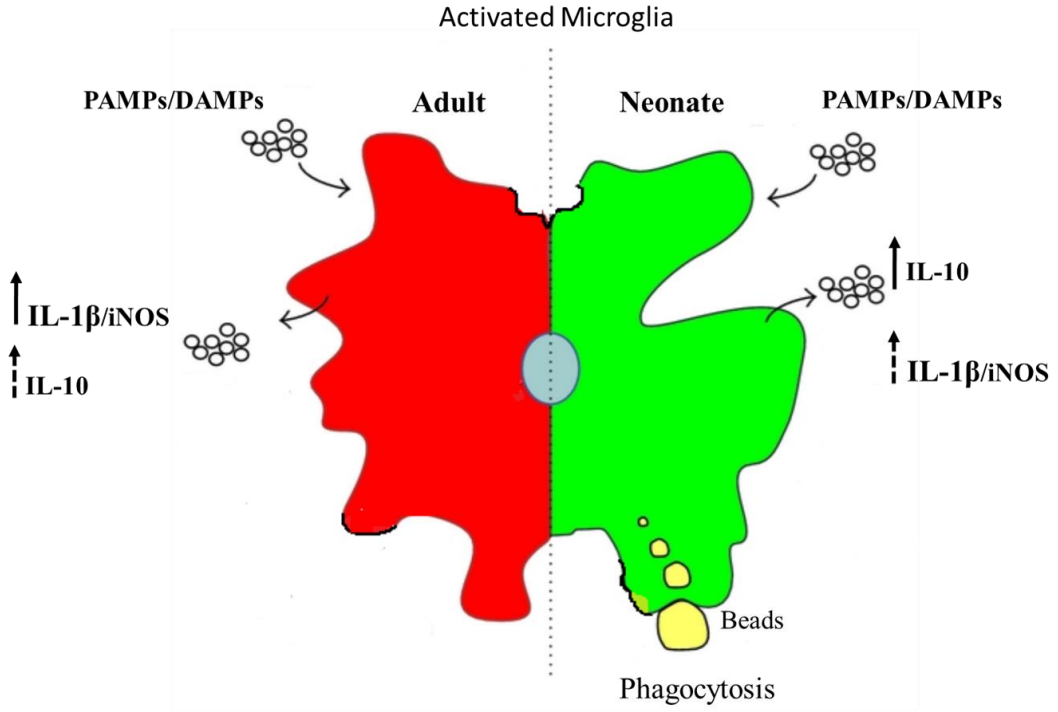


Figure 4.25. Proposed mechanism for the regulation of neonatal and adult microglia after immune activation. The activated neonatal microglia release several pro- and anti-inflammatory cytokines. In this model of DAMPs and PAMPs activation, neonatal microglia become stimulated and increase the expression of IL-10 and increased the microglia phagocytic activity. However, PAMPs and DAMPs stimulation induced expression of pro-inflammatory cytokines including IL-1β and iNOS in adult microglia. These toxic factors are suggested to act with neurotoxic factors released by microglia leading to neuronal death.

5 Chapter Five: Investigation of the anti-inflammatory role of the orphan nuclear receptor and transcription factor Nurr1 in microglia

5.1 Introduction

The NR4A family of nuclear receptors are early response regulators that have emerged as key regulators of inflammatory processes required in inflammatory diseases initiation and progression (Zhao and Bruemmer, 2010, McMorrow and Murphy, 2011). NR4A receptors play a pivotal role in orchestrating immune cell homeostasis through molecular crosstalk NF-kB (Bonta et al., 2006). Nur77 depletion promotes polarization of macrophage towards a pro-inflammatory phenotype following TLR and TNFα stimulation (Hamers et al., 2011). Recently, it has been shown that Nor1 knockdown in myeloid cells enhanced expression of NF-kB driven TNFα and MCP-1 (Crean et al., 2015).

Previous work on Nurr1 have revealed that this receptor is essential for the survival of dopaminergic neurons, and genetic deletion of Nurr1 in mice limits the development of mid-brain dopaminergic neurons in PD (Le et al., 2003, Saijo et al., 2013). The dopaminergic neurons in heterozygous ($Nurr1^{+/-}$) mice showed more susceptibility to exhibit high vulnerability to dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) more than those of wild-type animals (Le et al., 1999). Nurr1 also acts in midbrain astrocytes and microglia to modulate the inflammatory response that triggers the death of dopaminergic neurons (Saijo et al., 2009a). Nurr1 has also shown an important neuroprotective role in response to stress, as Nurr1 is induced by cAMP-response element binding protein (CREB) in neurons exposed to stressful insults (Volakakis et al., 2010) and Nurr1 is responsible for the NMDA-mediated increase in BDNF, which is necessary for the NMDA-mediated prosurvival effect on cerebellar granule cells (Barneda-Zahonero et al., 2012).

Nurr1 is a promising target for novel neuroprotective therapeutic targets of neurodegenerative diseases (Saijo et al., 2009a). It is well-known that reduced expression of Nurr1 caused death to TH-expressing neurons (Decressac et al., 2013). Farshbaf et al. (2016) revealed that Nurr1 agonists improved cell viability and reduced cell death rate and intracellular ROS of PD. In addition, Liu et al. (2017) found that Nurr1 over-expression showed anti-inflammatory effects by decreasing the release of pro-inflammatory mediators including, IL-1β and TNF-α, promoting viability *in vivo* and *in vitro*, and inhibiting apoptosis.

Several Nurr1 splice variants have been cloned previously from rat and human mid-brain (Michelhaugh et al., 2005). A Nurr1 splice variant, TINUR, transcriptionally inducible nuclear receptor (Okabe et al., 1995), is produced through an alternative splice site within exon 3, shortening the AF1 domain. Another alternative splice site in exon 7 produces the variants Nurr1a, which lacks the carboxy terminus (Castillo et al., 1997, Castillo et al., 1998b). Two additional Nurr1 variants were identified in rat and human, Nurr1b, which lacks 18 amino acids within the LBD but has intact carboxy-terminus (Ichinose et al., 1999, Michelhaugh et al., 2005), and Nurr2, which produces by utilization of exon 3 and exon 7 alternative splice sites, shortening both termini (Ohkura et al., 1999). These Nurr1 isoforms have been identified previously only in clonal cell lines or total brain cDNA libraries. Because of the crucial role of Nurr1 in development and maintenance of dopaminergic neurons, this study sought to define the expression of Nurr1 variants expressed in neonatal and adult primary microglia, and to assess their anti-inflammatory role when over-expressed in microglia.

Aims

Nurr1 plays a central role in regulating microglial and astrocytic phenotype. Nurr1 has been shown to inhibit expression of pro-inflammatory neurotoxic mediators by docking to NF-kB-p65 on target inflammatory gene promoters in microglia (Saijo et al., 2009b, Kim et al., 2015). These findings show that Nurr1 could be a promising therapeutic target in neuro-inflammatory diseases. However, the specific functions and/or mechanisms of Nurr1 in microglia during postnatal development are still unknown. In this Chapter, the potential role of Nurr1 or other members of the NR4A family in the observed differences in phenotype between neonate and adult microglia was investigated. Several splice variants of Nurr1 have been previously described in many studies (Castillo et al., 1998a, Michelhaugh et al., 2005). However, studies comparing their relative abundance and transcriptional activity in microglia are lacking. In this study, the expression of Nurr1 variants and their activity in microglia has been investigated. Reporter gene assays, Taqman qPCR and lentiviral expression vectors were used to explore the role of the Nurr1 transcription factor in inflammatory responses in neonatal and adult microglial cells.

5.2 Results

5.2.1 NR4A orphan nuclear receptor expression in microglia in response to inflammatory stimuli

Expression of NR4A nuclear receptors was screened in neonatal and adult microglia treated with LPS and ATP. Nurr1 mRNA was rapidly induced (after 1h) by LPS and ATP (3.21±0.26 and 7.50±0.66 fold, respectively) in neonatal spinal microglia (Figure 5.1A and C). Interestingly, the expression of Nurr1 decreased rapidly after LPS induction but remained elevated over 6h after ATP stimulation. Note that the level of Nurr1 expression in ATP-stimulated neonatal microglia was more than that in LPS-stimulated cells (7.50±0.66 vs 3.21±0.26 fold). ATP and LPS treatment of adult spinal microglia revealed that Nurr1 was potently induced at early time points (1h) only after ATP stimulation (13.47±2.65 fold) (Figure 5.1B and D).

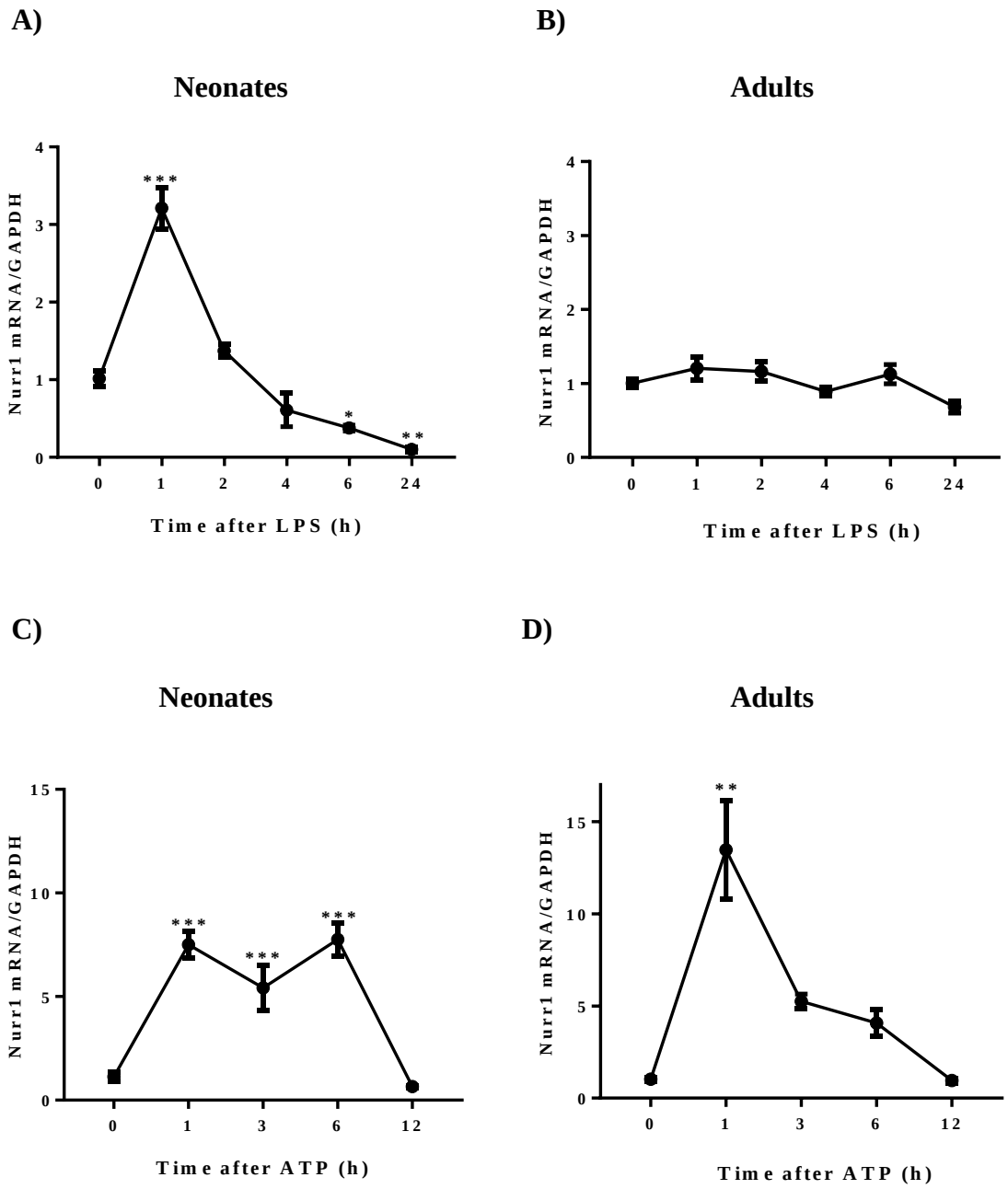


Figure 5.1. Expression of Nurr1 is induced by inflammatory stimuli in spinal microglia. Primary spinal microglia were isolated from rat pups and adults and their response to stimulation with LPS (100ng/ml) and ATP (100µM) measured using Taqman qPCR to measure changes in the expression of Nurr1 and normalized to GAPDH expression. Neonatal (A) and adult (B) microglial cells were treated with LPS for the indicated times (1-24h). Neonatal (C) and adult (D) microglial cells were treated with ATP for the indicated times (1-12h). The graphs represent means±SEM obtained from three independent repeat experiment. **, *** = P<0.01 and P<0.001 respectively, one-way ANOVA with Bonferroni post-tests.

Taqman qPCR analysis showed that Nur77 mRNA levels in neonatal spinal microglia elevated 1h after LPS and ATP stimulation (40.62±2.75 and 15.15±2.76 fold, respectively) (Figure 5.2A and C). A similar pattern was also observed in adult spinal microglia, the LPS and ATP induced Nur77 mRNA rapidly (after 1h) (2.37±0.35 and 28.00±4.31 fold, respectively) (Figure 5.2B and D). Note that the mRNA levels of Nur77 in LPS-stimulated neonatal microglia was more than that in ATP-stimulated cells (40.62±2.75 vs 15.15±2.76 fold), but mRNA levels of Nur77 in ATP-stimulated adult microglia was massively more than that in LPS-stimulated cells (28.00±4.31 vs 2.37±0.35 fold).

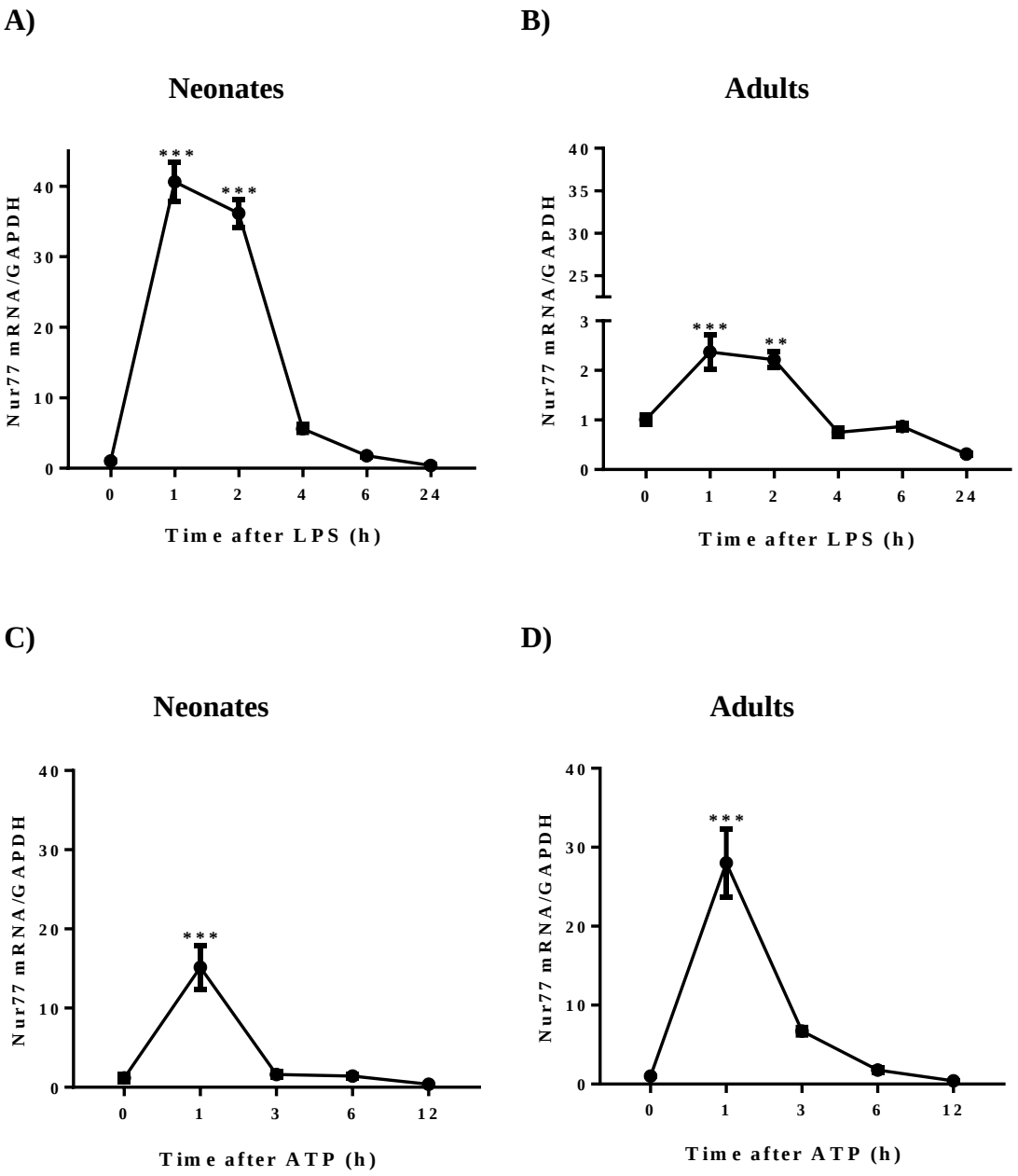


Figure 5.2. Expression of Nur77 is increased after inflammatory stimuli exposure in spinal microglia. Primary spinal microglia were isolated from rat pups and adults, and their response to stimulation with LPS (100ng/ml) and ATP (100µM) measured using Taqman qPCR to measure changes in the expression of Nur77 and normalized to GAPDH expression. Neonatal (A) and adult (B) microglial cells were treated with LPS for the indicated times (1-24h). Neonatal (C) and adult (D) microglial cells were treated with ATP for the indicated times (1-12h). The graphs represent means±SEM obtained from three independent repeat experiment. **, *** = P<0.01 and P<0.001 respectively, one-way ANOVA with Bonferroni post-tests.

Expression of Nor1 was found to be highly induced in neonatal spinal microglial cells in response to treatment with LPS or ATP. Nor1 mRNA transcript levels peaked at 1h post-ATP (38.40±10.25 fold), and increased significantly 2h after LPS treatment (34.05±4.02 fold), but peaked at 4h (48.62±6.11 fold) (Figure 5.3A and C). Expression of Nor1 mRNA increased after early (1h) ATP induction (29.05±3.13) in adult spinal microglia, whereas Nor1 mRNA transcript levels increased over the time course of 24h in these cells after LPS treatment (Figure 5.3B and D). Consistent with the results obtained from the expression of Nurr1 and Nur77 in adult spinal microglia, Nor1 mRNA expression in ATP-stimulated adult microglia was much greater than that in LPS- stimulated cells.

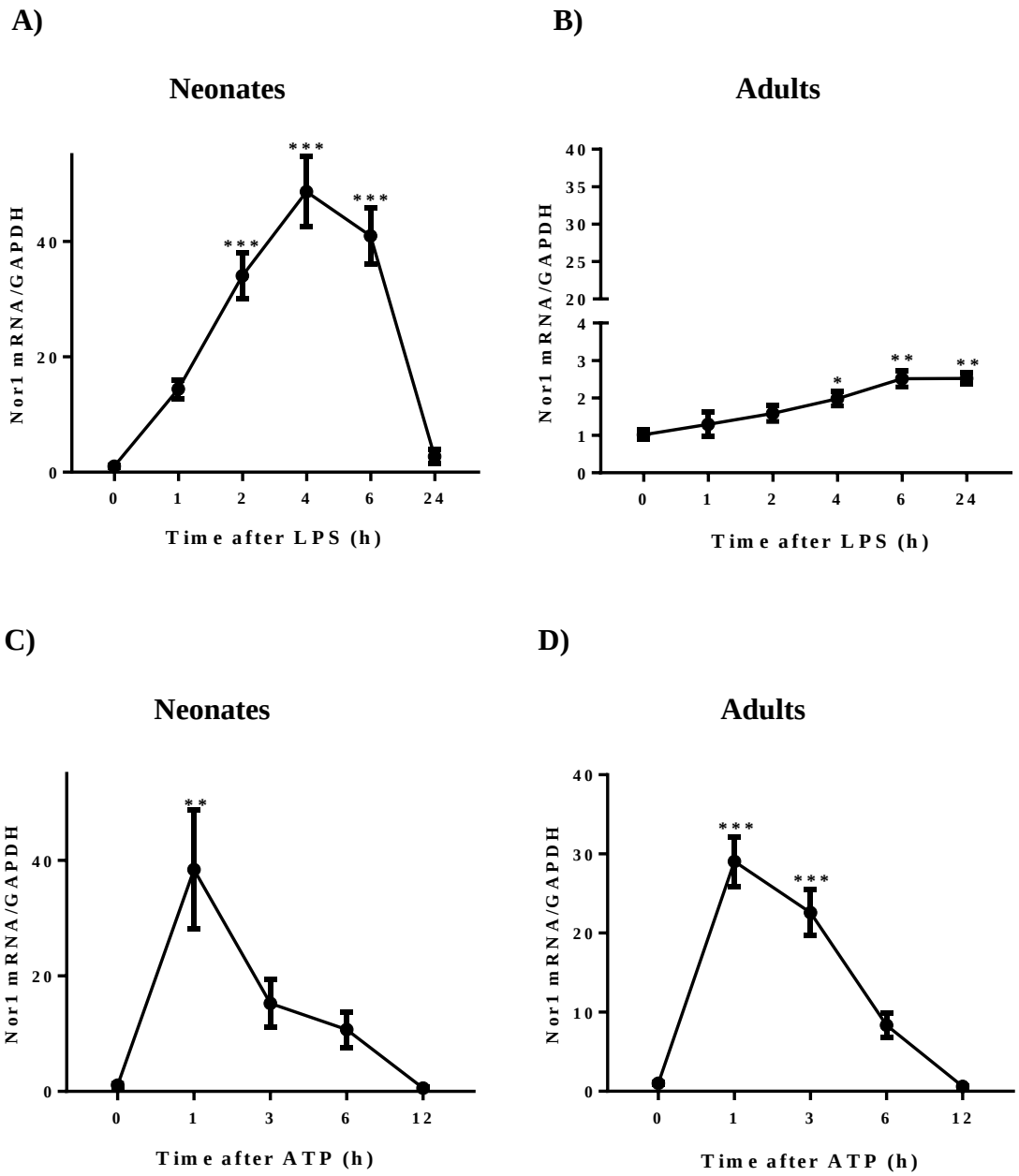


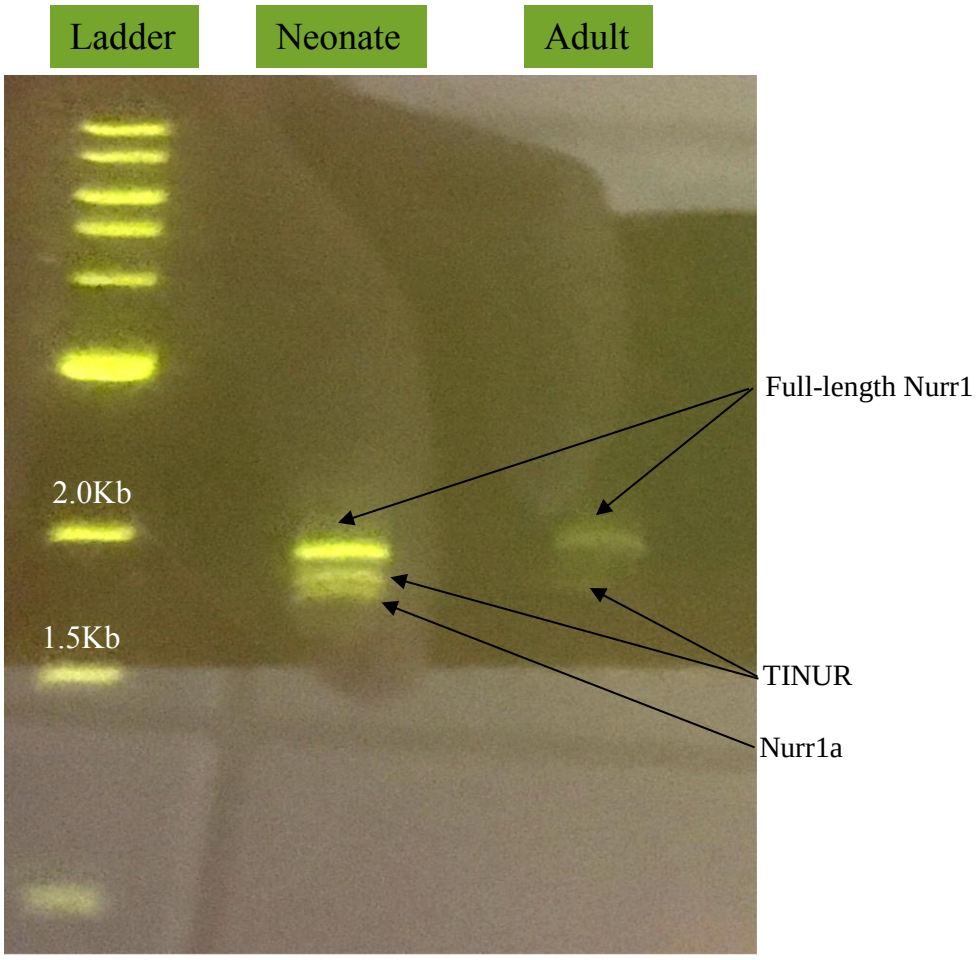
Figure 5.3. Expression of Nor1 is induced by inflammatory stimuli in spinal microglia. Primary spinal microglia were isolated from rat pups and adults and their response to stimulation with LPS (100ng/ml) and ATP (100µM) measured using Taqman qPCR to measure changes in the expression of Nor1 and normalized to GAPDH expression. Neonatal (A) and adult (B) microglial cells were treated with LPS for the indicated times (1-24h). Neonatal (C) and adult (D) microglial cells were treated with ATP for the indicated times (1-12h). The graphs represent means±SEM obtained from three independent repeat experiment. **, *** = P<0.01 and P<0.001 respectively, one-way ANOVA with Bonferroni post-tests.

The findings of the present work have revealed that the expression of Nur77, NOR1, and Nurr1 is highly inducible in microglia by diverse inflammatory stimuli, including LPS and ATP. As Nurr1 has been implicated in anti-inflammatory processes in microglia (Saijo et al., 2009a), it was decided to concentrate on that particular member of the family.

5.2.2 Multiple variants of Nurr1 identified in neonatal and adult microglia

In order to explore which splice variants were expressed in neonatal and adult microglial cells of the spinal cord, a set of PCR primers matching common sequences in exon 2 and exon 8 which span the whole coding region of full-length Nurr1 capable of amplifying all of its splice variants were designed (see section 2.11.9 in materials and methods chapter). As shown in Figure 5.4A, multiple distinct bands were identified by gel electrophoresis and ethidium bromide staining in both neonatal and adult microglia. Individual bands were extracted from the gel and subsequently cloned and sequenced. The sequencing results revealed that full-length Nurr1, Nurr1a and TINUR were all detected in neonatal microglia, whereas only full-length Nurr1 and TINUR were expressed in adult microglia. Next we examined microglial Nurr1 protein expression in response to LPS. Western blot analysis using an antibody that detect Nurr1 variants showed that in neonatal microglia two variants could be detected at the protein level, whereas adult microglia expressed only one variant at a level detectable by western blot (Figure 5.4B).

A)



B)

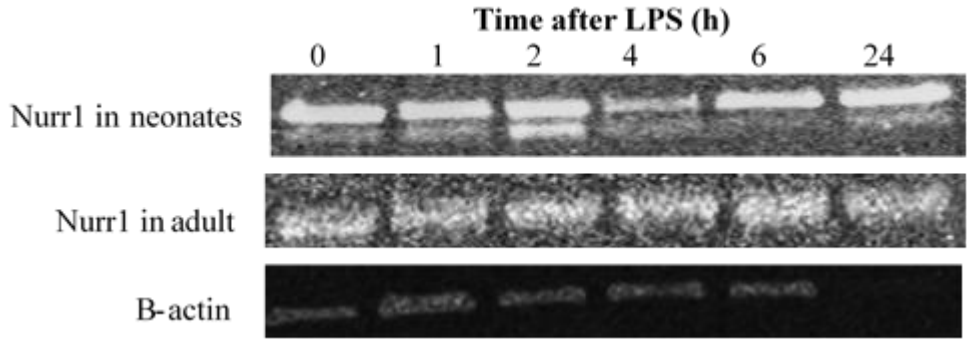
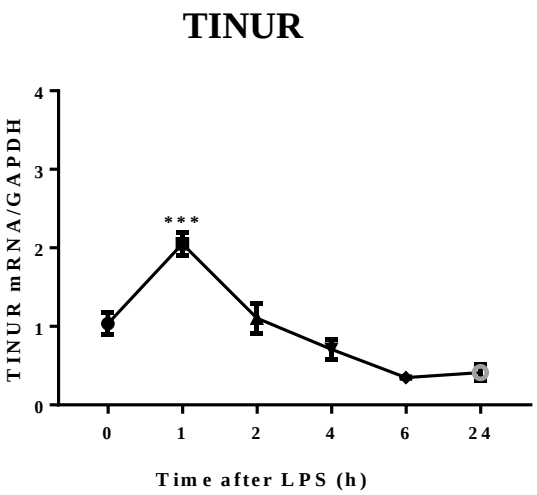


Figure 5.4. Multiple Nurr1 splice variants are identified in neonatal and adult microglia. A) RNA from neonatal and adult microglia was subjected to RT-PCR using specific primers designed to amplify the entire Nurr1 coding region. Multiple distinct bands were identified by gel electrophoresis and ethidium bromide staining. The neonatal microglia express three different variants Full-length Nurr1, TINUR and Nurr1 whereas adult microglia express only two Nurr1 variants full-length Nurr1 and TINUR. B) Western blot of Nurr1 protein in microglia after the treatment with LPS (100ng/ml). MW represents molecular weight.

5.2.3 Nurr1 variant expression in neonatal microglia in response to LPS and ATP

In order to determine the relative expression of two splice variants TINUR and Nurr1a in microglia after LPS and ATP exposure, Taqman qPCR was carried out with specific primers and probes for each variant. As shown in Figure 5.1, the Nurr1 (full-length) mRNA transcript level was rapidly upregulated (after 1h) by LPS in neonatal microglia. This set of Taqman primers and probes for Nurr1 would detect all of the splice variants. In addition, the cloning data was revealed that unstimulated neonatal microglia expressed three Nurr1 variants; full-length Nurr1, TINUR, and Nurr1a. Therefore, the mRNA expression levels of TINUR and Nurr1a were investigated in neonatal microglia upon LPS stimulation using specific Primers and probe set for each variants. In neonatal microglia, TINUR mRNA transcript levels increased rapidly (after 1h) upon LPS stimulation (2.05±0.15 fold, $P<0.01$) (Figure 5.5A). However, Nurr1a mRNA expression levels increased also rapidly (1.75±0.26 fold) after LPS exposure, but this trend did not reach statistical significance (Figure 5.5B).

A)



B)

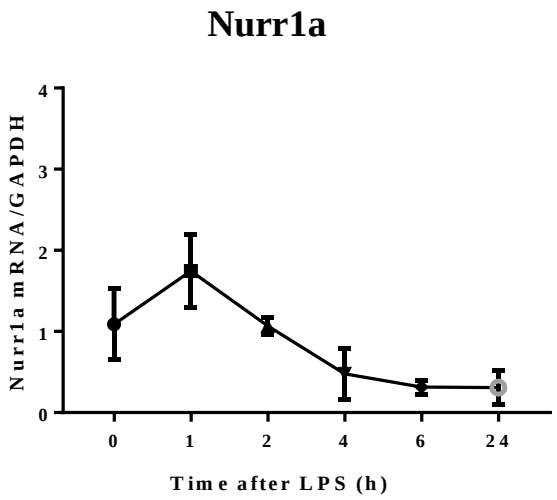


Figure 5.5. Expression of Nurr1 variants are induced by LPS in neonatal microglia. Primary spinal microglia were isolated from rat pups and adults and their response to stimulation with LPS (100ng/ml) measured using Taqman qPCR to measure changes in the expression of Nurr1 variants TINUR (A) and Nurr1a (B), and normalized to GAPDH expression. Neonatal microglial cells were treated with LPS for the indicated times (1-24h). The mRNA expression of TINUR increased significantly after 1h of LPS treatment. The graphs represent means±SEM obtained from three repeats. *** = P<0.001, one-way ANOVA with Bonferroni post-tests.

5.2.4 Nurr1 variants expression in neonatal and adults microglia in response to ATP

As shown in the Figure 5.1, Nurr1 mRNA expression increased over the course of 6 h in neonatal microglia upon ATP stimulation. A similar pattern was observed for TINUR mRNA expression, as mRNA expression increased over the course of 6h in neonatal microglia (4.51±0.33; $P<0.01$, 3.36±0.60; $P<0.05$, 5.47±0.45 fold; $P<0.001$, after 1h, 3h and 6h respectively) (Figure 5.6A). However, Nurr1a mRNA transcript levels increased over the course of 6h in neonatal microglia after ATP exposure, but this trend statistically not significant (Figure 5.6C). A time course of ATP treatment of adult microglia revealed that TINUR mRNA expression were strongly induced at early time point (after 1h) (7.44±1.44 fold, $P<0.001$) (Figure 5.6B).

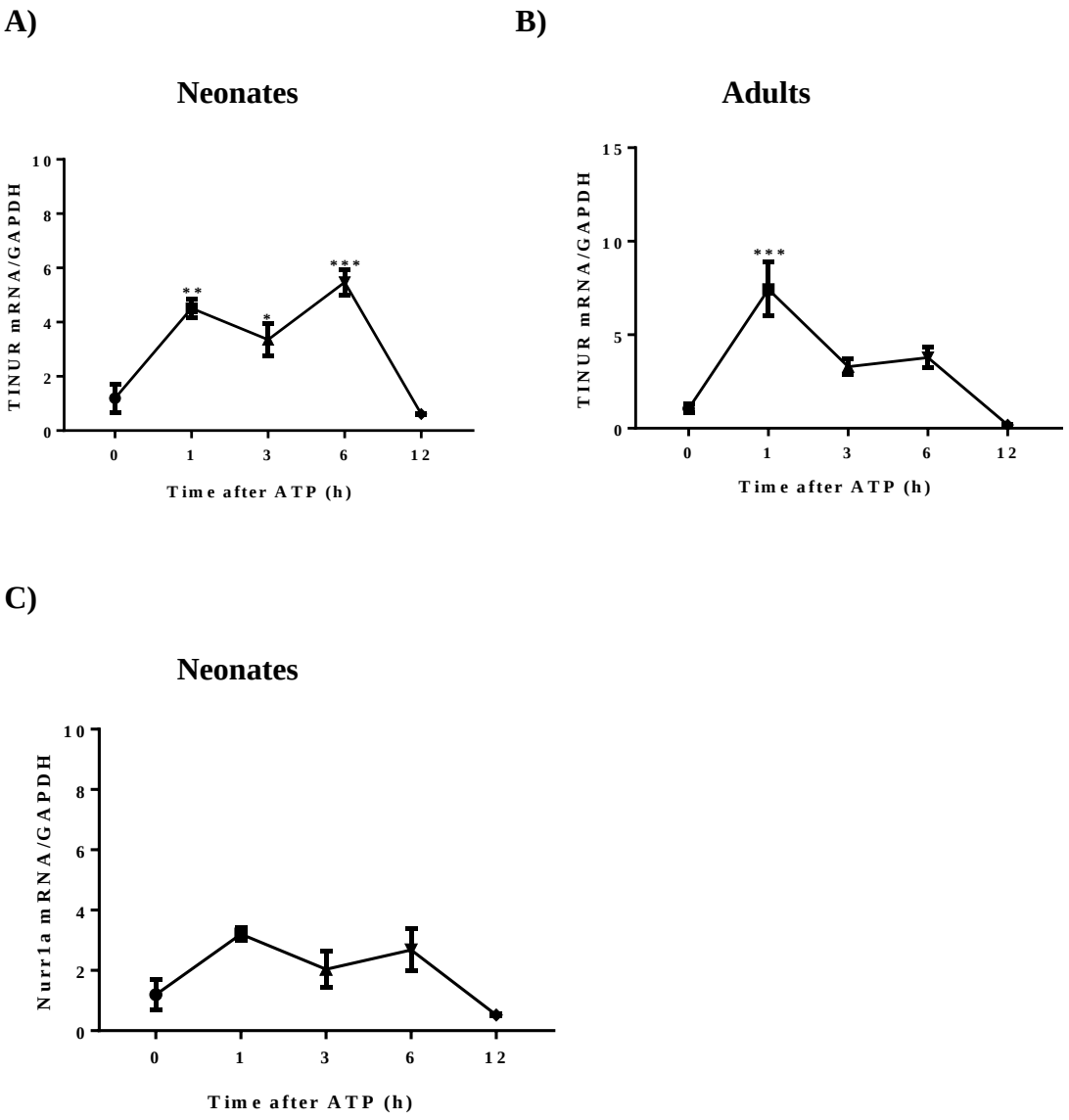
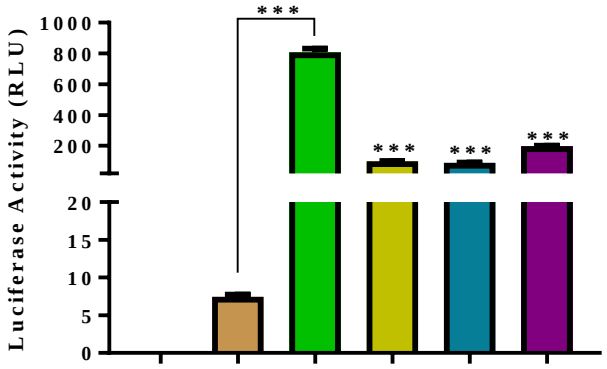


Figure 5.6. Expression of Nurr1 variants are induced by ATP in neonatal and adult microglia. Primary spinal microglia were isolated from rat pups and adults and their response to stimulation with ATP (100µM) measured using Taqman qPCR to measure changes in the expression of Nurr1 variants TINUR in neonatal (A) and adult microglia (B) and Nurr1a (C) in adult microglia, and normalized to GAPDH expression. Neonatal microglial cells were treated with LPS for the indicated times (1-12h). TINUR mRNA levels increased significantly after 1h of ATP exposure in both neonatal and adult microglia, whereas Nurr1a increased not significantly after ATP exposure in neonatal microglia. The graphs represent means±SEM obtained from three repeats. *, **, *** = $P<0.05$, $P<0.01$ and $P<0.001$ respectively, one-way ANOVA with Bonferroni post-tests.

5.2.5 The trans-repressive effects of Nurr1 variants on iNOS promoter activity

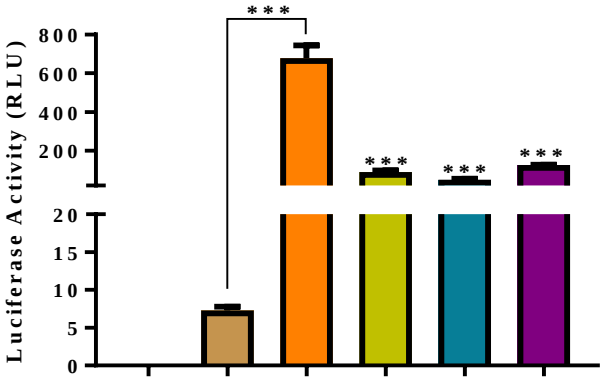
The effect of Nurr1 on trans-repression of iNOS promoter was studied by luciferase reporter gene assay. iNOS was used because it is produced by macrophages and its expression is controlled by inflammatory inducers such as LPS and ATP (Lechner et al., 2005). Raw264.7 mouse macrophage cell line was transiently transfected with iNOS promoter driven reporter plasmid directing luciferase expression was transfected together with the empty expression vector (pcDNA3.1/Zeo+) or co-transfection with full-length Nurr1/Nurr1a/TINUR expression vectors and stimulated with LPS, ATP or HMGB1. Raw cells were chosen because they contain all the components required for inflammatory signalling such as TLR4. Our findings showed that the full-length Nurr1, TINUR and Nurr1a were able to trans-repress LPS-driven transcription of the iNOS promoter (Figure 5.7A). Similarly, the full-length Nurr1, TINUR and Nurr1a were able to trans-repress ATP- and HMGB1-driven transcription of the iNOS promoter (Figure 5.7B and C).

(A)



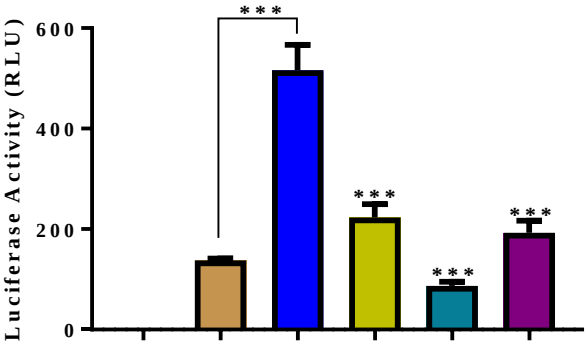
pcDNA3.1 (vector)	+	+	-	-	-	-
iNOS promoter	-	+	+	+	+	+
FL Nurr1 construct	-	-	-	+	-	-
TINUR	-	-	-	-	+	-
Nurr1a	-	-	-	-	-	+
LPS	-	-	+	+	+	+

(B)



pcDNA3.1 (vector)	+	+	-	-	-	-
iNOS promoter	-	+	+	+	+	+
FL Nurr1 construct	-	-	-	+	-	-
TINUR	-	-	-	-	+	-
Nurr1a	-	-	-	-	-	+
ATP	-	-	+	+	+	+

(C)



pcDNA3.1 (vector)	+	+	-	-	-	-
iNOS promoter	-	+	+	+	+	+
FL Nurr1 construct	-	-	-	+	-	-
TINUR	-	-	-	-	+	-
Nurr1a	-	-	-	-	-	+
HMGB1	-	-	+	+	+	+

Figure 5.7. Nurr1 isoforms mediate the inhibition of LPS-, ATP- and HMGB1-induced iNOS gene expression in macrophage. Raw264.7 cells were transfected with 500ng iNOS and pcDNA3.1-/Zoe(+) or full-length Nurr1 or TINUR or Nurr1a expression vector 500ng each, using X-tremeGENE HP DNA transfection reagent (3:1 w/w X-tremeGENE: DNA). 24hrs later, cells were treated with 100ng/ml LPS or 100µM ATP or 250ng/ml HMGB1. After 24 hours cells were lysed by using passive lysis buffer and luciferase activity was measured in transfected lysate. Results are expressed as relative luminometric units (RLU), considering empty vector induced by LPS or ATP or HMGB1 as a control. The results obtained from three independent experiments (n=3per group). Data were analysed with one way ANOVA, with Bonferroni's multiple comparison post-hoc test. The graphs represent means-±SEM compared to iNOS promoter+LPS/ATP/HMGB group (*, **, *** = P<0.05, P<0.01 and P<0.001 respectively).

5.2.6 The effects of Nurr1 variants on IL-10 promoter activity

To assess the ability of Nurr1 isoforms to activate transcription of the IL-10 promoter, Raw264.7 mouse macrophage cell line was transiently transfected with IL-10 promoter driven reporter plasmid directing luciferase expression was transfected together with the empty expression vector (pcDNA3.1/Zoe+) or co-transfection of full-length Nurr1/Nurr1a/TINUR expression vectors; and with or without stimulation with LPS, ATP or HMGB1. In Raw264.7 cells, full-length Nurr1 and TINUR caused significant activation of the transcription of IL-10 promoter, while Nurr1a did not (Figure 5.8.). In addition, both full-length Nurr1 and TINUR caused significant activation of LPS-driven transcription of the IL-10 promoter, while Nurr1a did not (see Figure 5.9A). Interestingly, only TINUR appeared to be able to significantly activate the ATP- and HMGB1-driven expression of the IL-10 promoter transcription (Figure 5.9B and C).

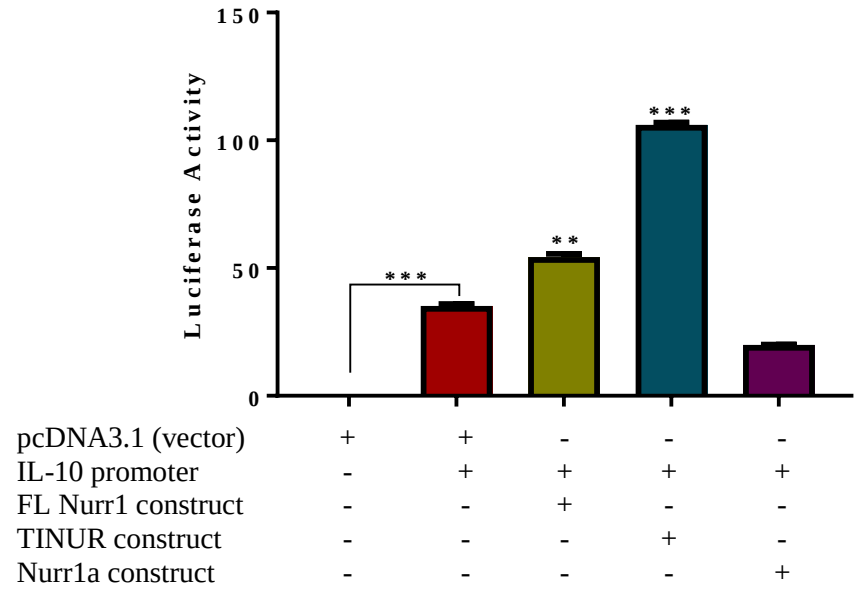
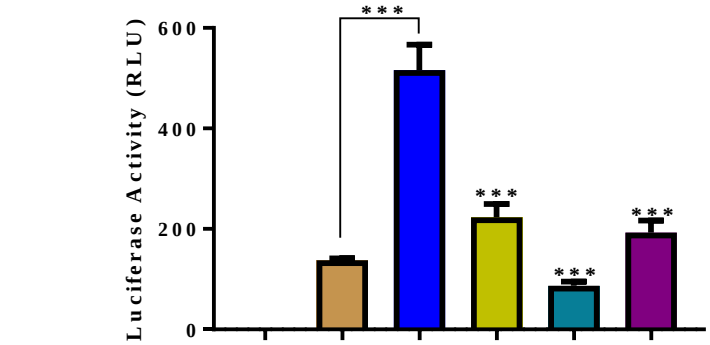


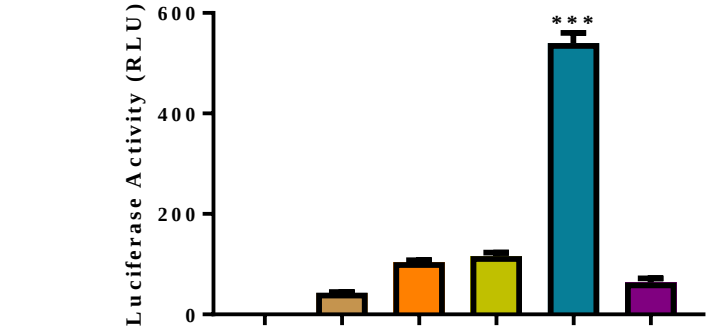
Figure 5.8. Nurr1 isoforms mediate the activation of IL-10 gene expression in macrophage. Raw264.7 cells were transfected with 500ng IL-10 and pcDNA3.1/Zoe(+) or full-length Nurr1 or TINUR or Nurr1a expression vector 500ng each, using X-tremeGENE HP DNA transfection reagent (3:1 w/w X-tremeGENE: DNA). After 24 hours cells were lysed by using passive lysis buffer and luciferase activity was measured in transfected lysate. Results are expressed as relative luminometric units (RLU), considering empty vector as a control. The results obtained from three independent experiments (n=3 per group). Data were analysed with one way ANOVA, with Bonferroni's multiple comparison post-hoc test. The graphs represent means±SEM compared to IL-10 promoter group (*, **, *** = $P<0.05$, $P<0.01$ and $P<0.001$ respectively).

(A)



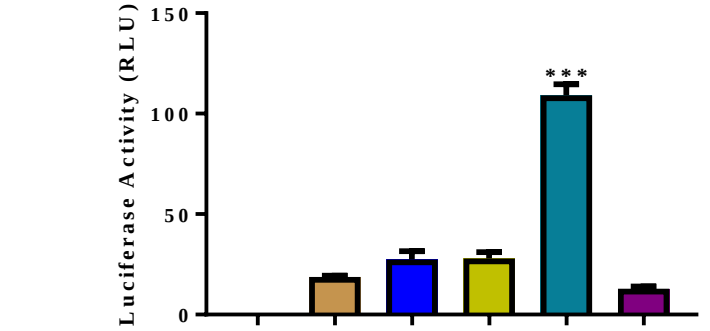
pcDNA3.1 (vector)	+	+	-	-	-	-
IL-10 promoter	-	+	+	+	+	+
FL Nurr1 construct	-	-	-	+	-	-
TINUR	-	-	-	-	+	-
Nurr1a	-	-	-	-	-	+
LPS	-	-	+	+	+	+

(B)



pcDNA3.1 (vector)	+	+	-	-	-	-
IL-10 promoter	-	+	+	+	+	+
FL Nurr1 construct	-	-	-	+	-	-
TINUR	-	-	-	-	+	-
Nurr1a	-	-	-	-	-	+
ATP	-	-	+	+	+	+

(C)



pcDNA3.1 (vector)	+	+	-	-	-	-
IL-10 promoter	-	+	+	+	+	+
FL Nurr1 construct	-	-	-	+	-	-
TINUR	-	-	-	-	+	-
Nurr1a	-	-	-	-	-	+
HMGB1	-	-	+	+	+	+

Figure 5.9. Nurr1 isoforms mediate the activation of LPS-, ATP- and HMGB1-induced IL-10 gene expression in macrophage. Raw-264.7 cells were transfected with 500ng IL-10 and pcDNA3.1/Zoe(+) or full-length Nurr1 or TINUR or Nurr1a expression vector 500ng each, using X-tremeGENE HP DNA transfection reagent (3:1 w/w X-tremeGENE: DNA). 24hrs later, cells were treated with 100ng/ml LPS or 100µM ATP or 250ng/ml HMGB1. After 24hrs cells were lysed by using passive lysis buffer and luciferase activity was measured in transfected lysate. Results are expressed as relative luminometric units (RLU), considering empty vector induced by LPS or ATP or HMGB1 as a control. The results obtained from three independent experiments (n=3 per group). Data were analysed with one way ANOVA, with Bonferroni's multiple comparison post-hoc test. The graphs represent means±SEM compared to IL-10 promoter+ LPS/ATP/HMGB1 group (*, **, *** = P<0.05, P<0.01 and P<0.001 respectively).

5.2.7 Effects of Lentiviral expression of Nurr1 variants in microglia

5.2.7.1 Lentivirus packaging and infection efficiency

The ability of pINDUCER20 lentiviral constructs to infect and express cloned cDNA in microglia was determined by infection with a GFP lentivirus construct. Fluorescence microscopy indicated >80% of cells expressed GFP as shown in Figure 5.11.

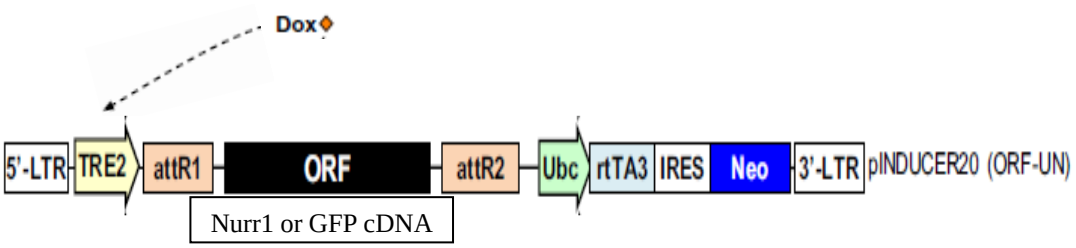


Figure 5.10. pInducer20 lentivirus elicits inducible Nurr1 and GFP expression. Diagram of the pInducer20 vector which allows easy cloning of (Nurr1-variants or GFP) cDNAs from Gateway entry vectors (see Materials and Methods chapter). Following the addition of doxycycline (dox), transcription of the cDNA is activated.

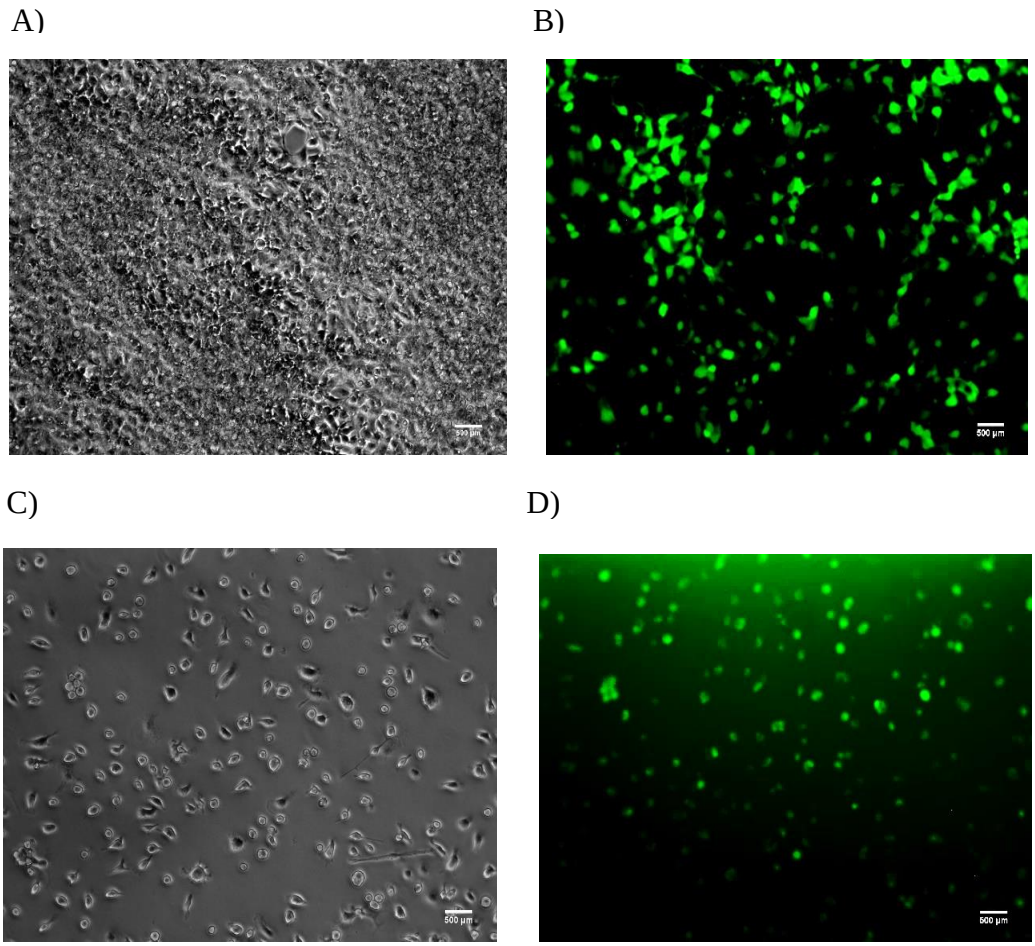
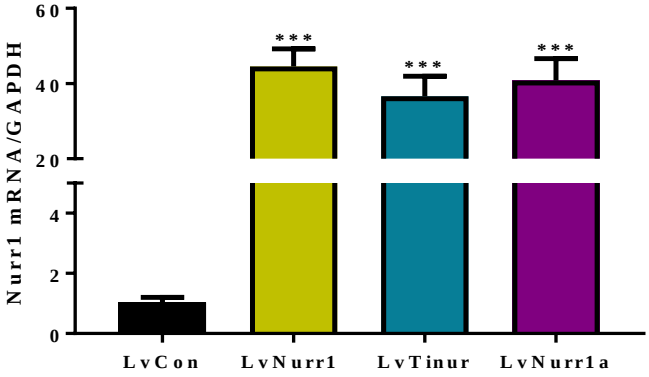


Figure 5.11. Green fluorescent (GFP) expression in HEK293FT cells and in rat microglia following lentiviral infection. Lentivirus was generated as described in methods section 2.15. HEK293FT cells and microglia were viewed 48h following the induction by doxycycline. (A) Bright field image of HEK293FT. (B) Expression of GFP in the same area viewed by fluorescence. (C) Bright field image of microglia (D) Expression of GFP in the same area viewed by fluorescence.

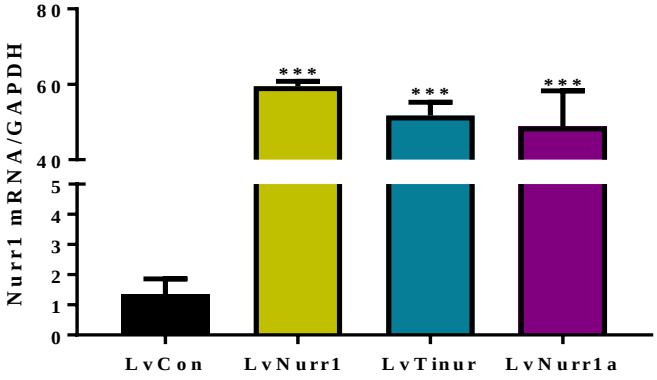
5.2.7.2 mRNA level and protein expression of Nurr1 variants in microglia following infection with Nurr1-carrying lentivirus

To confirm the lentiviral infection efficiency, the Nurr1 variants expression in neonatal and adult microglia was analysed by Taqman qPCR, followed by Western blotting using a Nurr1-specific antibody. Nurr1 lentivirus infection led to strongly increased mRNA expression levels of Nurr1 variants in both neonatal and adult microglia (neonates (full-length-Nurr1: 44.57±4.67, TINUR: 36.63±5.34, Nurr1a: 40.81±5.79) (Figure 5.12A); adults (full-length Nurr1: 59.52±1.30, TINUR: 51.73±3.52, Nurr1a: 48.85±9.43 Figure 5.12B). Western blotting was carried out to determine the levels of Nurr1 variant protein in lentivirus infected cells and control. As shown in Figure 5.12C, The Nurr1 protein expression levels increased following the infection with Nurr1-carrying lentivirus. However, the Nurr1 antibody did not detect the Nurr1a variant.

(A)



(B)



(C)

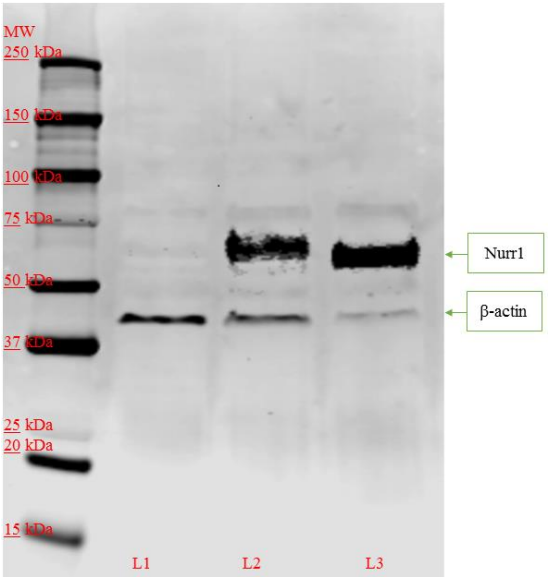


Figure 5.12. Nurr1 overexpression efficiency. Lentivirus was generated using the standard production and concentration protocol (see the materials and methods chapter). Neonatal and adults microglial cells, which were infected with lentivirus loaded with Nurr1 variants or control lentivirus for 4h then induced with doxycycline for 48h. A. Effects of lentivirus used in this study were verified by qPCR and Western blotting for their respective targets. A. Nurr1 mRNA levels increased in neonatal microglia following the infection with nurr1-carrying lentivirus. B. Nurr1 mRNA levels increased in adult microglia following the infection with Nurr1-carrying lentivirus. Protein levels of Nurr1 in each cell type are shown in bottom panel. Data Bonferroni's multiple comparison post-hoc test. The graphs represent means±SEM obtained from three repeats. *, **, *** = P<0.05, P<0.01 and P<0.001 respectively. (C) Western blot of Nurr1 protein in microglia after the infection with Nurr1-carrying lentivirus. MW represents molecular weight. L1 represents control cells infected with LvCon, L2 represent cells infected with LvNurr1 and L3 represent cells infected with LvTinur. LvCon: Lentivirus carrying empty plasmid, LvNurr1: Lentivirus carrying full-length Nurr1, LvTinur: Lentivirus carrying TINUR, LvNurr1a: Lentivirus carrying Nurr1a.

5.2.7.3 Effects of Lentiviral expression of Nurr1 variants in adult microglia

As shown in Chapter Four, the adult microglial cells display a pro-inflammatory phenotype characterised by significant increases in IL-1β and iNOS following PAMP and DAMP exposure. In addition, Taqman RT-PCR and Western blot results displayed that Nurr1 mRNA and protein were increased rapidly upon PAMP and DAMP stimulation in microglia, and interestingly the luciferase assay revealed that the Nurr1 variants were able to trans-repress the inflammatory response and trans-activate the anti-inflammatory response as well. TINUR over-expression in adult microglia caused a significant increase in IL-10 mRNA in the absence of inflammatory stimulation, whereas full- length Nurr1 and Nurr1a did not (Figure 5.13.). Based on these observations, the consequences of over-expression of Nurr1 in neonatal and adult microglia after PAMP and DAMP stimuli was assessed. Increasing the expression of Nurr1 variants in adult microglia using lentivirus expressing Nurr1 variants led to significant decrease in ATP-induced expression of inflammatory mediators, including IL-1β (full-length Nurr1: -8.99±0.08, TINUR: -8.79±0.18 and Nurr1a: -8.45±0.12; $P<0.01$) and iNOS (full-length Nurr1: -16.73±2.12, TINUR: -17.49±1.67 and Nurr1a: -17.38±1.85; $P<0.05$) relative to control lentivirus (Figures 5.14A and B).

To explore whether increased expression of Nurr1 in adult microglia resulted in activation of anti-inflammatory mediators, Taqman qPCR experiment was performed to measure the IL-10 mRNA levels following ATP exposure in Nurr1-overexpressed adult microglia. Interestingly, Taqman qPCR results revealed that the IL-10 mRNA levels increased significantly only in TINUR-overexpressed adult microglia (3.4±0.35, $P<0.001$) after ATP stimulation, whereas no significant increase in IL-10

mRNA levels in full-length Nurr1- and Nurr1a-overexpressed adult microglia after ATP exposure (1.34±0.14 and 1.41±0.19 respectively) (Figure 5.14C).

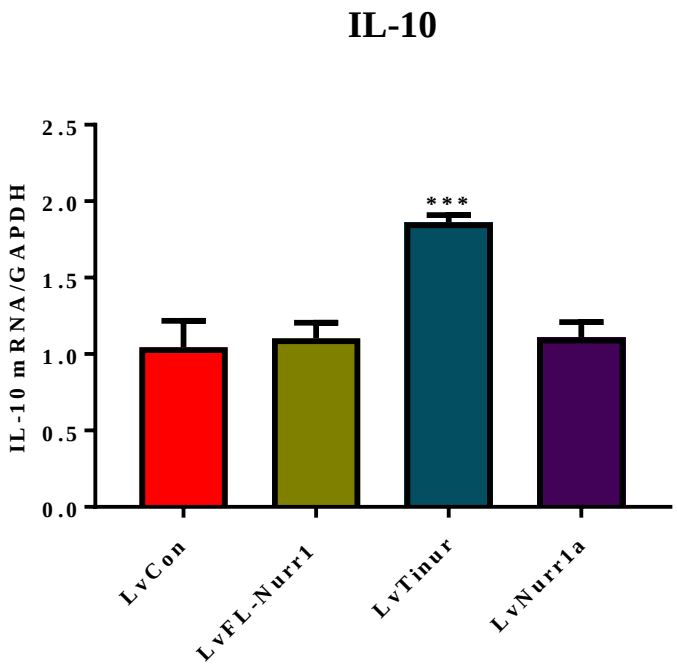
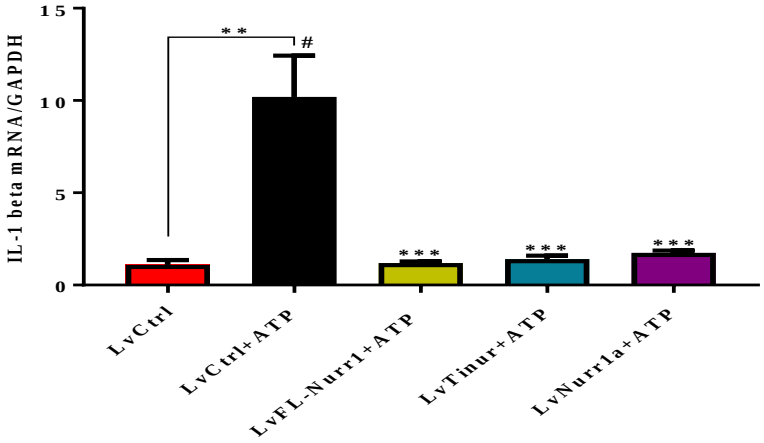


Figure 5.13. TINUR trans-activates IL-10 in adult microglia. Lentivirus was generated using the standard production and concentration protocol (see the materials and methods chapter). Adults microglial cells, which were infected with lentivirus loaded with Nurr1 variants or control lentivirus for 4h then transduced with doxycycline for 48h. Expression of IL-10 mRNA in Nurr1 variants-overexpression cells determined by qPCR and normalised to GAPDH expression (n=4). Data represent mean±SEM. *** = P<0.001 respectively, one-way ANOVA with Bonferroni post-tests. LvCtrl: Lentivirus carrying empty plasmid, LvFL-Nurr1: Lentivirus carrying full-length Nurr1, LvTinur: Lentivirus carrying TINUR, LvNurr1a: Lentivirus carrying Nurr1a.

(A)



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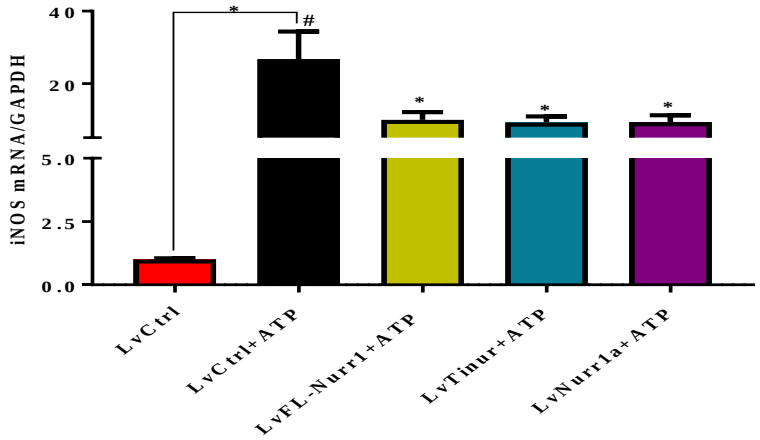

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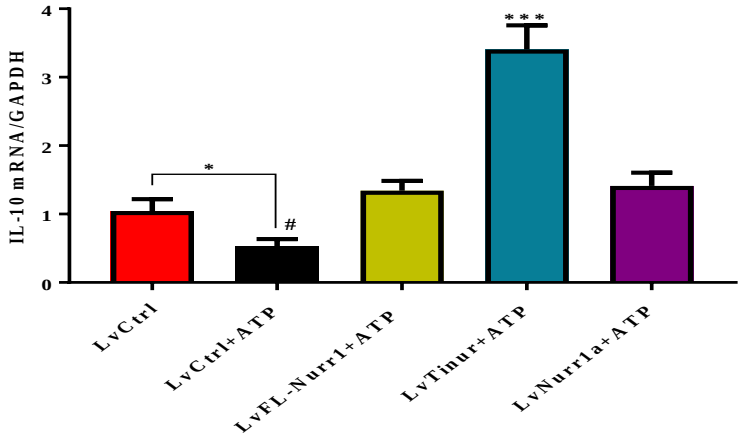
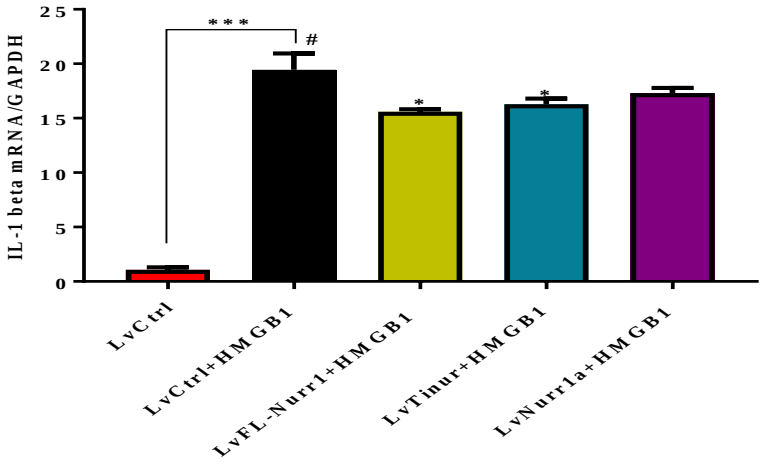


Figure 5.14. Nurr1 suppresses ATP-induced inflammation in adult microglia. Lentivirus was generated using the standard production and concentration protocol (see the materials and methods chapter). Adults microglial cells, which were infected with lentivirus loaded with Nurr1 variants or control lentivirus for 4h then transduced with doxycycline for 48h. Expression of IL-1β (A) iNOS (B) and IL-10 (C) mRNA in Nurr1 variants-overexpression cells 12 hr after ATP (100μM) treatment as determined by qPCR and normalised to GAPDH expression (n=4). Data represent mean±SEM. # represents the control (LvCtrl/ATP). LvCtrl: Lentivirus carrying empty plasmid, LvFL-Nurr1: Lentivirus carrying full-length Nurr1, LvTinur: Lentivirus carrying TINUR, LvNurr1a: Lentivirus carrying Nurr1a. *, **, *** = P<0.05, P<0.01 and P<0.001 respectively, one-way ANOVA with Bonferroni post-tests.

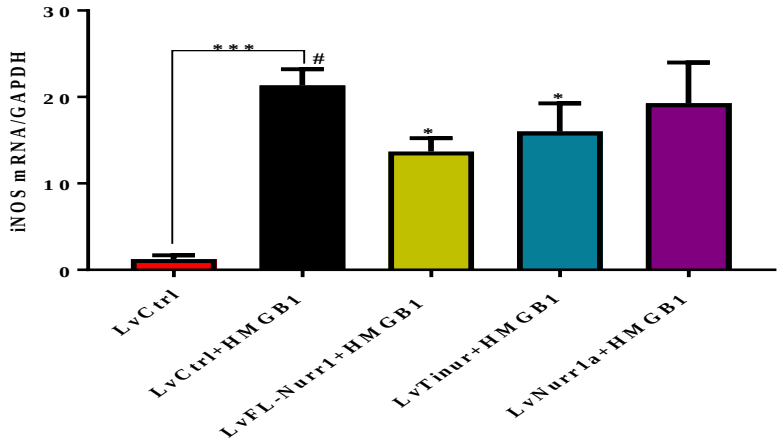
Adult microglial cells infected with Nurr1 variants expressing lentivirus, followed by HMGB1 treatment for 12h. Overexpression of full-length Nurr1 and TINUR appeared to be able to decrease the mRNA expression of the inflammatory mediators including, IL-1β (-3.86±0.23 and -3.16±0.50 respectively, relative to control lentivirus, $P<0.05$) and iNOS (-7.63±1.53 and -5.35±2.29 respectively, $P<0.05$) in HMGB1-induced adult microglia. However, overexpression of Nurr1a variant was not able to significantly supress the inflammatory mediators, IL-1β and iNOS, in HMGB1-stimulated adult microglia (-2.14±0.48 and -2.07±0.72, respectively) (Figure 5.15A and B).

Increased expression of TINUR in HMGB1-induced adult microglia using TINUR-carrying lentivirus showed a trend to increase mRNA expression level of anti-inflammatory cytokine IL-10 (0.78±0.05), but this trend was not statistically significant. However, increased expression of the other variants, full-length Nurr1 and Nurr1a, were not able to increase the mRNA expression level of IL-10 in HMGB1-stimulated adult microglia (no detectable changes relative to control lentivirus) (Figure 5.15C).

(A)



(B)



(C)

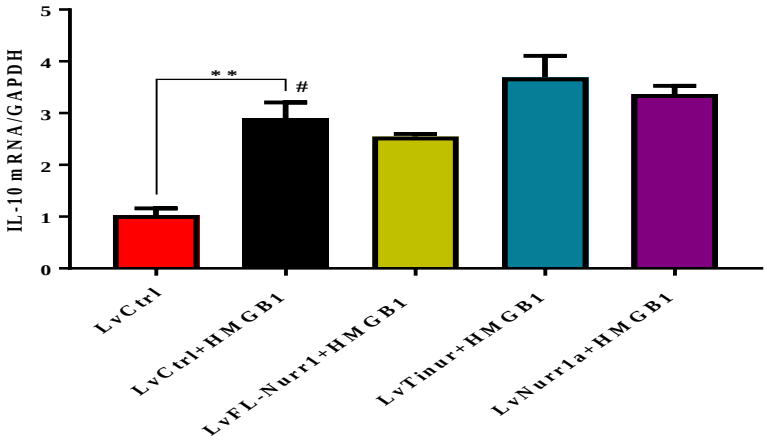


Figure 5.15. Full-length Nurr1 and TINUR suppress HMGB1-induced inflammation in adult microglia. Lentivirus was generated using the standard production and concentration protocol (see the materials and methods chapter). Adults microglial cells, which were infected with lentivirus loaded with Nurr1 variants or control lentivirus for 4hrs then transduced with doxycycline for 48h. Expression of IL-1β (A) iNOS (B) and IL-10 (C) mRNA in Nurr1 variants-overexpression adult microglia 12hr after HMGB1 (500ng/ml) treatment as determined by qPCR and normalised to GAPDH expression (n = 4). Data represent mean±SEM. **, *** = P<0.01 and P<0.001 respectively, one-way ANOVA with Bonferroni post-tests. # represents the control (LvCtrl/HMGB1). LvCtrl: Lentivirus carrying empty plasmid, LvFL-Nurr1: Lentivirus carrying full-length Nurr1, LvTinur: Lentivirus carrying TINUR, LvNurr1a: Lentivirus carrying Nurr1a.

The overexpression of Nurr1 variants was not able to either repress the inflammatory mediators, including IL-1β and iNOS (no detectable changes relative to control lentivirus) (Figure 5.16A and B), or activate the anti-inflammatory cytokine IL-10 (no detectable change relative to control lentivirus) in LPS-induced adult microglia (Figure 5.16C).

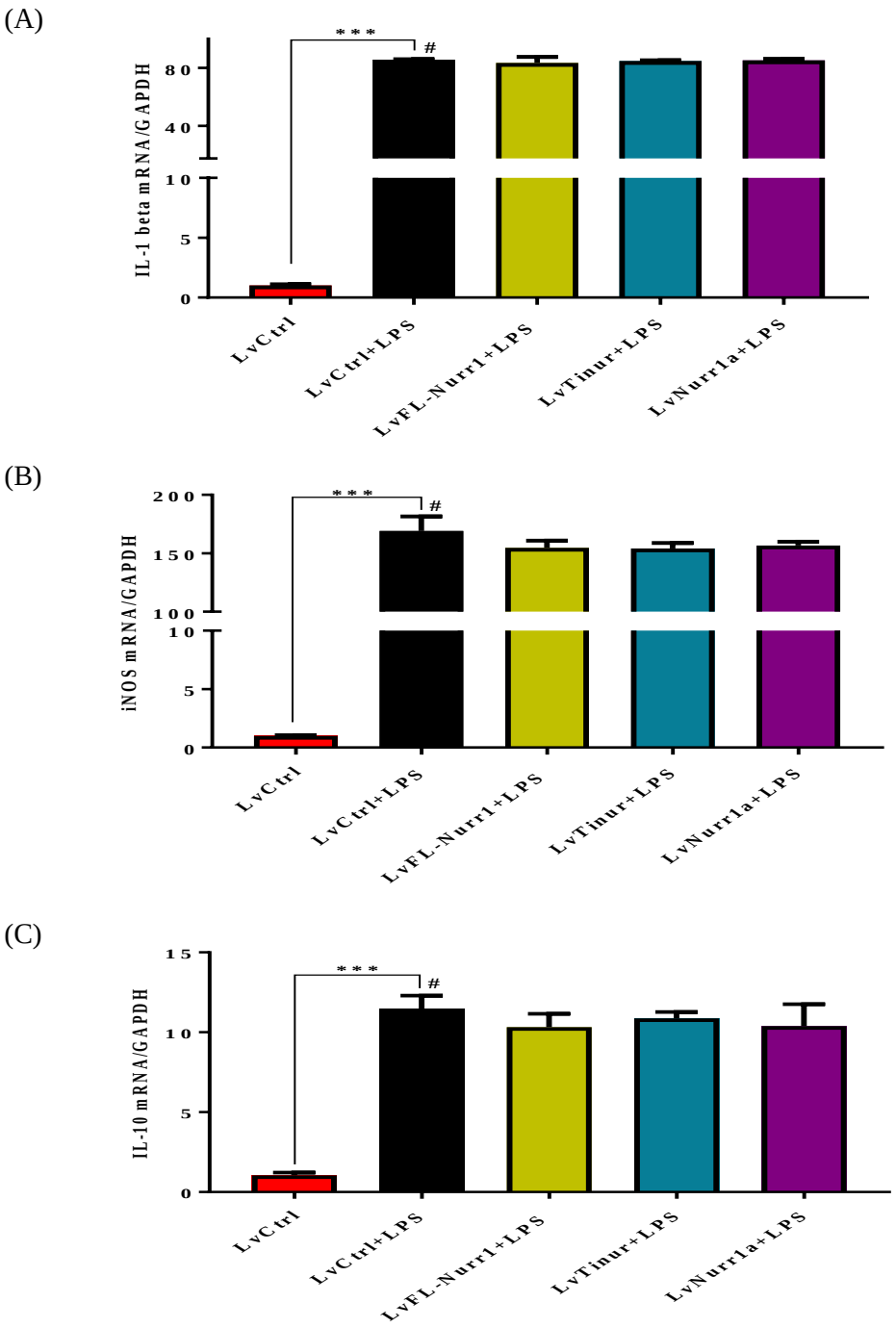


Figure 5.16. Nurr1 was not able to suppress LPS-induced inflammation in adult microglia. Lentivirus was generated using the standard production and concentration protocol (see the materials and methods chapter). Adults microglial cells, which were infected with lentivirus loaded with Nurr1 variants or control lentivirus for 4h, then transduced with doxycycline for 48hrs. Expression of IL-1β (A) iNOS (B) and IL-10 (C) mRNA in Nurr1 variants-overexpression adult microglia 6hr after LPS (100ng/ml) treatment as determined by Taqman qPCR and normalised to GAPDH expression (n=4). Data represent mean ±SEM. *** = P<0.001 respectively, one-way ANOVA with Bonferroni post-tests. # represents the control (LvCtrl/LPS). LvCtrl: Lentivirus carrying empty plasmid, LvFL-Nurr1: Lentivirus carrying full-length Nurr1, LvTinur: Lentivirus carrying TINUR, LvNurr1a: Lentivirus carrying Nurr1a.

5.2.7.4 Effects of Lentiviral expression of Nurr1 variants in neonatal microglia

To test whether increase the expression of Nurr1 in neonatal microglia resulted in induction of an anti-inflammatory phenotype, lentivirus-expressing Nurr1 variants were introduced into neonatal microglia. Over-expression of full-length Nurr1 and TINUR resulted in significant increase in mRNA expression of IL-10 in neonatal microglia (Figure 5.17).

The mRNA expression level of IL-1β was decreased to the basal levels in cells infected with lentivirus expressing Nurr1 variants after ATP activation compared to control cells (LvCtrl+ATP) (full length-Nurr1: -0.66±0.14, TINUR: -0.69±0.08 and Nurr1a: -0.20±0.02) (Figure 5.18A).

A significant decrease of iNOS mRNA expression levels (-3.90±0.60, $P<0.05$) in TINUR-overexpressing neonatal microglia was observed after ATP stimulation relative to control lentivirus whereas full length Nurr1 and Nurr1a had no significant effect (Figure 5.18B).

Figure 5.18C shows that TINUR-overexpressing neonatal microglia showed a marked increase in IL-10 mRNA (5.63±1.36, $P<0.01$) relative to control lentivirus following ATP stimulation. Whereas Nurr1- and Nurr1a- overexpression in neonatal microglia had no significant effect.

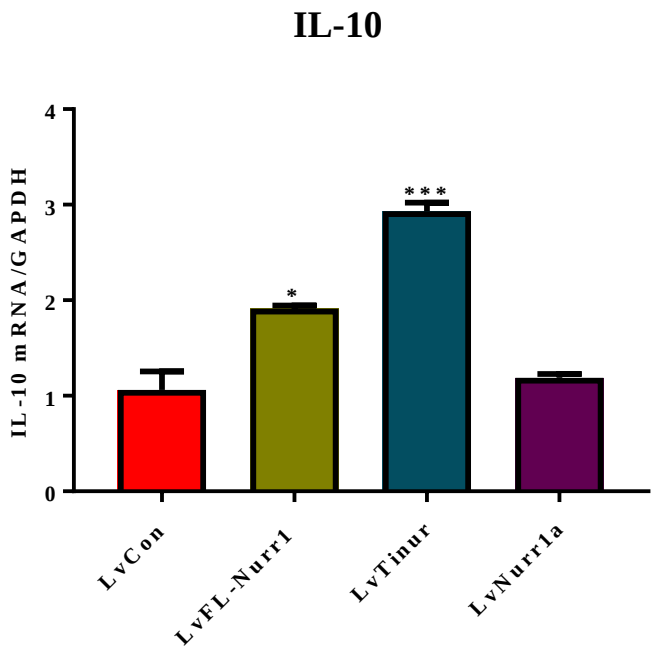
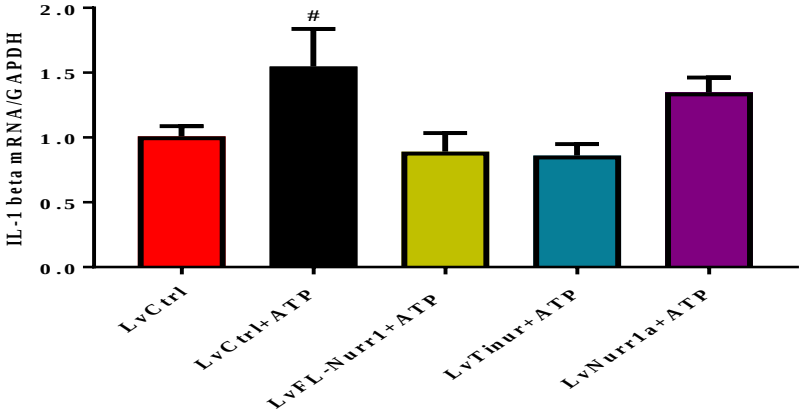


Figure 5.17. TINUR trans-activates IL-10 in neonatal microglia. Lentivirus was generated using the standard production and concentration protocol (see the materials and methods chapter). Neonatal microglial cells, which were infected with lentivirus loaded with Nurr1 variants or control lentivirus for 4h then transduced with doxycycline for 48h. Expression of IL-10 mRNA in Nurr1 variants-overexpression cells determined by qPCR and normalised to GAPDH expression (n=4). Data represent mean±SEM. *, *** = P<0.05 and P<0.001 respectively, one-way ANOVA with Bonferroni post-tests. LvCtrl: Lentivirus carrying empty plasmid, LvFL-Nurr1: Lentivirus carrying full-length Nurr1, LvTinur: Lentivirus carrying TINUR, LvNurr1a: Lentivirus carrying Nurr1a.

(A)



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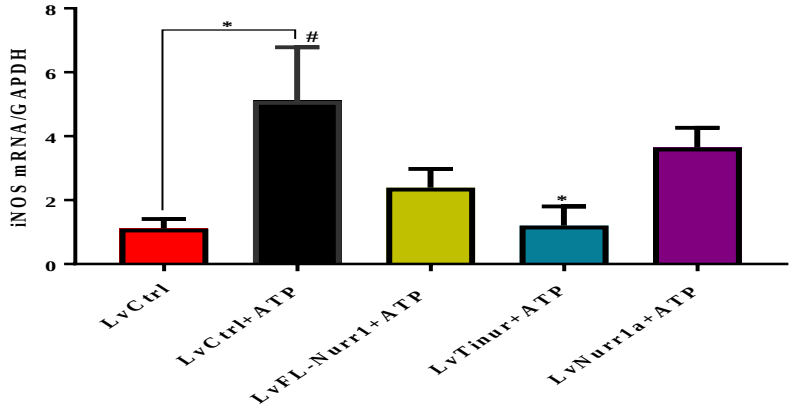


(C)

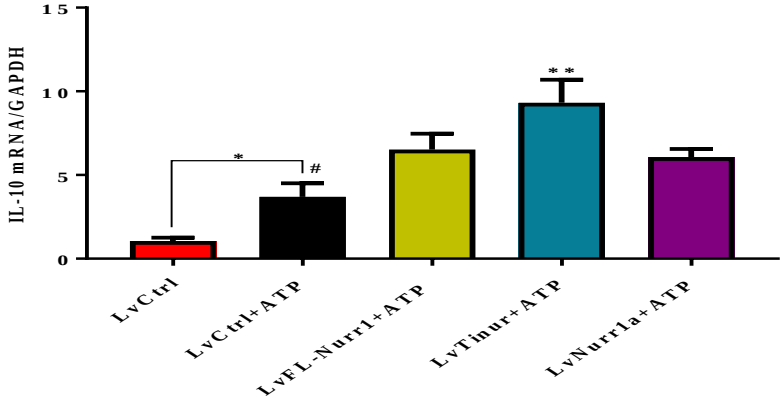
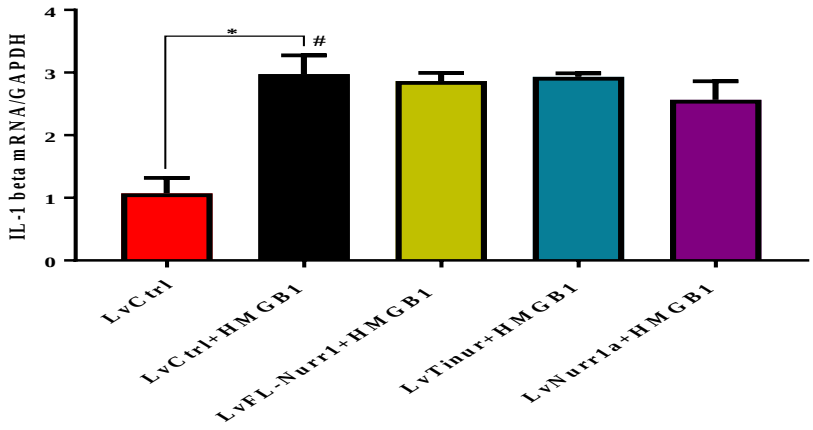


Figure 5.18. Nurr1 was able to induce anti-inflammatory activity in ATP-induced neonatal microglia. Lentivirus was generated using the standard production and concentration protocol (see the materials and methods chapter). Neonatal microglial cells, which were infected with lentivirus loaded with Nurr1 variants or control lentivirus for 4h then transduced with doxycycline for 48hrs. Expression of IL-1β (A) iNOS (B) and IL-10 (C) mRNA in Nurr1 variants-overexpression neonatal microglia 12h after ATP (100μM) treatment as determined by qPCR and normalised to GAPDH expression (n=4). Data represent mean±SEM. *,** = P<0.05 and P<0.01, one-way ANOVA with Bonferroni post-tests. # represents the control (LvCtrl/ATP). LvCtrl: Lentivirus carrying empty plasmid, LvFL-Nurr1: Lentivirus carrying full-length Nurr1, LvTinur: Lentivirus carrying TINUR, LvNurr1a: Lentivirus carrying Nurr1a.

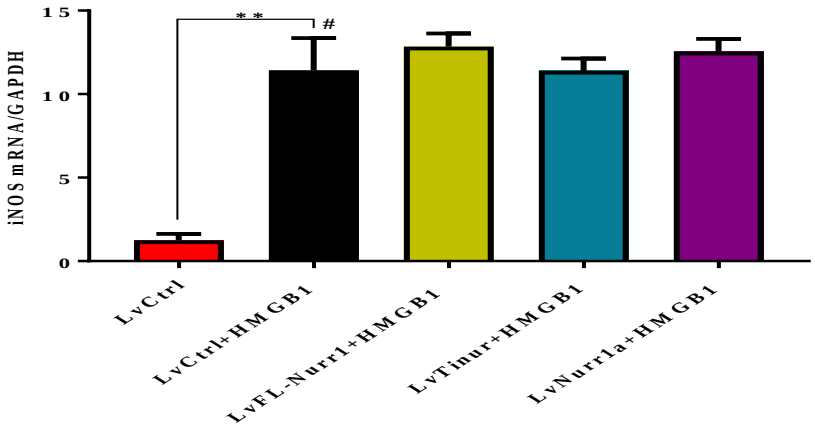
In contrast to ATP-induced neonatal microglia, the overexpression of Nurr1 variants in HMGB1-induced neonatal microglia showed no detectable changes in IL-1β and iNOS (Figure 5.19A and B), or IL-10 relative to control lentivirus (Figure 5.19C).

Lentiviral overexpression of Nurr1 variants had no significant effects upon cytokine gene expression following LPS activation in neonatal microglia (Figure 5.20A). (Figure 5.20B and C).

(A)



(B)



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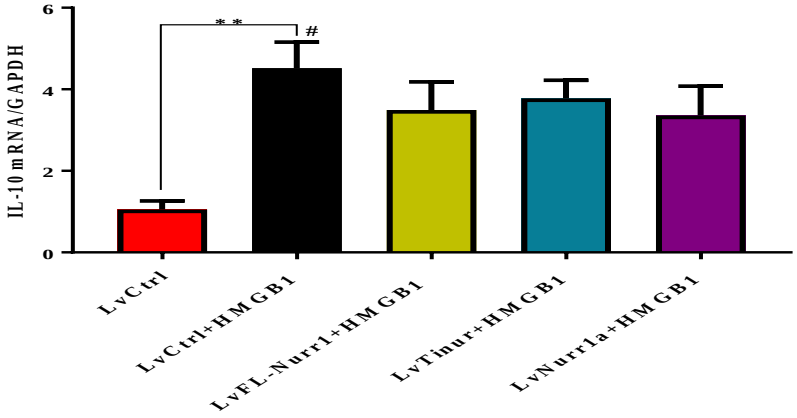
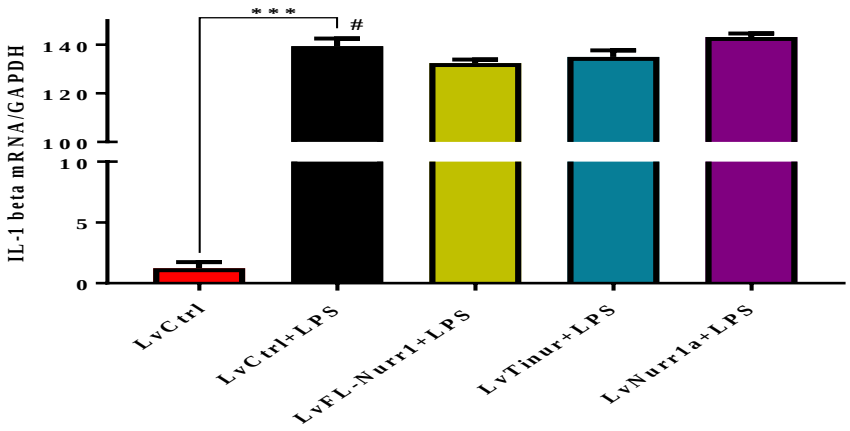
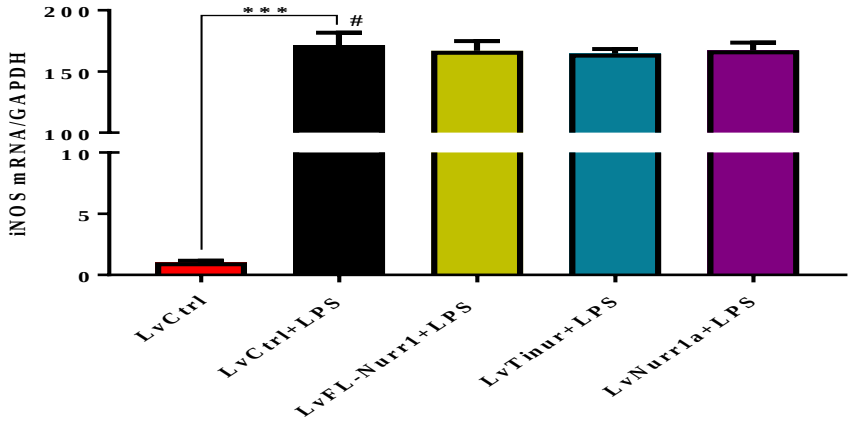


Figure 5.19. Nurr1 was not able to induce anti-inflammatory activity in HMGB1-induced neonatal microglia. Lentivirus was generated using the standard production and concentration protocol (see the materials and methods chapter). Neonatal microglial cells, which were infected with lentivirus loaded with Nurr1 variants or control lentivirus for 4h then transduced with doxycycline for 48hrs. Expression of IL-1β (A) iNOS (B) and IL-10 (C) mRNA in Nurr1 variants-overexpression neonatal microglia 12h after HMGB1 (500ng/ml) treatment as determined by qPCR and normalised to GAPDH expression (n=4). Data represent mean ± SEM. *, ** = P<0.05 and P<0.01 respectively, one-way ANOVA with Bonferroni post-tests. # represents the control (LvCtrl/HMGB1). LvCtrl: Lentivirus carrying empty plasmid, LvFL-Nurr1: Lentivirus carrying full-length Nurr1, LvTinur: Lentivirus carrying TINUR, LvNurr1a: Lentivirus carrying Nurr1a.

(A)



(B)



(C)

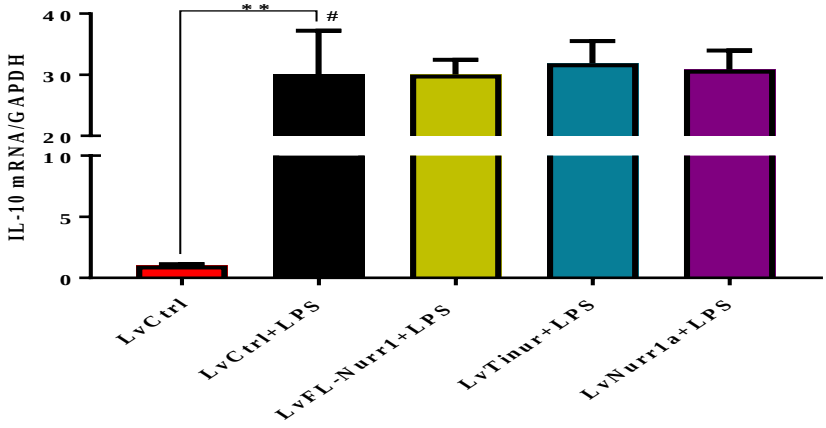


Figure 5.20. Nurr1 was not able to induce anti-inflammatory activity in LPS-induced neonatal microglia. Lentivirus was generated using the standard production and concentration protocol (see the materials and methods chapter). Adults microglial cells, which were infected with lentivirus loaded with Nurr1 variants or control lentivirus for 4h then transduced with doxycycline for 48hrs. Expression of IL-1β (A) iNOS (B) and IL-10 (C) mRNA in Nurr1 variants-overexpression neonatal microglia 6hr after LPS (100ng/ml) treatment as determined by qPCR and normalised to GAPDH expression (n=4). Data represent mean ± SEM. **, *** = P<0.01 and P<0.001 respectively, one-way ANOVA with Bonferroni post-tests. # represents the control (LvCtrl/LPS). LvCtrl: Lentivirus carrying empty plasmid, LvFL-Nurr1: Lentivirus carrying full-length Nurr1, LvTinur: Lentivirus carrying TINUR, LvNurr1a: Lentivirus carrying Nurr1a.

5.3 Discussion

The trans-repression effects of Nurr1 on neuro-inflammation in microglia and astrocytes have gained increasing attention in recent years. Saijo et al. (2009a) suggested that Nurr1 is linked to the regulation expression of neuro-inflammatory mediators in glial cells. The current study showed that Nurr1 overexpression exerted anti-inflammatory properties by decreasing the pro-inflammatory genes in both neonatal and adult microglia after ATP exposure.

The NR4A receptors were recognised originally as immediate-early response genes stimulated by varied inflammatory stimuli, including cytokines and TLR ligands in macrophage (Pei et al., 2005). In the current work, the expression of Nurr1, Nur77 and Nor1 in both neonatal and adult microglia was elevated in response to inflammatory stimuli, LPS and ATP (Figure 5.1). Nurr1 expression has been shown to be rapidly induced in neonatal microglial cells after exposure with inflammatory mediators. However, the expression of Nurr1 in adult microglia is induced only after ATP challenge, but not following LPS treatment. Thus, these findings suggest that Nurr1 may play different role in microglia inflammatory response over development.

To investigate the expression of Nurr1 variants in microglia, we employed PCR and cloning methods that would primarily target microglia to amplify and clone all expressed Nurr1 splice variants. Interestingly, this work is the first ever report that neonatal microglia expressed three Nurr1 splice variants: full-length Nurr1, TINUR and Nurr1a, whereas adult microglia expressed only two Nurr1 splice variants full-length Nurr1 and TINUR.

Nurr1 transcription activity is mediated by AF1, ligand binding domain (LBD) and AF2 domains (Wärnmark et al., 2003). As discussed earlier, the difference in the TINUR and Nurr1a isoforms is that they are produced by alternative splicing events. TINUR is lacking a part of the AF1 domain, while Nurr1a lacks 121bp from C-terminal, resulting in a deletion within LBD/AF2 (Figure 5.21).(Michelhaugh et al., 2005). In this chapter, all Nurr1 isoforms were able to trans-repress the PAMPs- and DAMPs-induction of the iNOS promoter in Raw 264.7 cells. Data showed that TINUR is the only isoform able to trans-activate the DAMPs-induction of the IL-10 promoter. However, Nurr1a was not able to trans-activate either PAMP-induced or DAMP-induced IL-10 promoter activity. Nurr1 like its related nuclear receptors Nur77 and Nor1, binds as a monomer or homodimer to the DNA binding sequence (NRBE) (Castro et al., 1999, Sacchetti et al., 2001). Furthermore, Nurr1 also activates transcription by binding with RXR and forms heterodimers (Castro et al., 1999). Characterization of the RXR-Nurr1 DNA interaction properties indicated that RXR-Nurr1 heterodimers bind to the response element (Perlmann and Jansson, 1995). However, Perlmann and Jansson (1995) reported that Nurr1 C-terminal is essential for RXR-Nurr1 heterodimer formation. Moreover, deletion of Nurr1 AF2 region prevented the formation of efficient RXR-Nurr1 heterodimers (Sacchetti et al., 2002).

In order to explore the potential role of Nurr1 in microglia inflammatory response, attempts to evaluate the impact of reducing Nurr1 expression in primary microglia cells have been made using different type of siRNAs (see chapter 2). Unfortunately, the siRNAs did not work in primary cells. Although many studies have reported that they could reduce Nurr1 expression using either siRNAs or short hairpin RNA (shRNA) against Nurr1 in microglial cell lines such as BV2 (Saijo et al., 2009a, De

Miranda et al., 2015, Spathis et al., 2017). Therefore, we produced lentivirus expressing Nurr1 to increase the expression of Nurr1 in primary microglial cells instead of the knockdown.

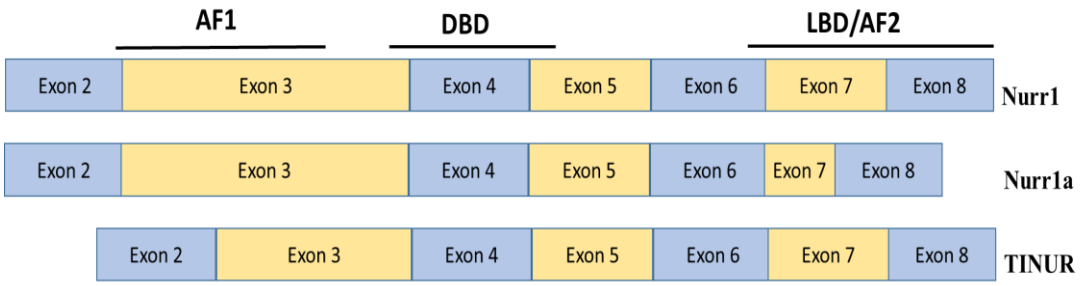


Figure 5.21. The schematic diagram shows rat Nurr1 isoforms functional domain structures. Full-length nurr1 has an activation function 1 (AF1) domain, a DNA-binding domain (DBD) and a ligand binding/activation function 2 (LBD/AF2) domain as indicated. TINUR isoform produced from alternative spicing within AF1 domain, and therefore missing 63 amino acids from the AF1 domain. Nurr1a isoform produced from alternative splicing within LBD/AF2, and therefore lacks 140 amino acids.

As mentioned in chapter 4, adult microglia displayed a pro-inflammatory phenotype which was characterized by an increase in the mRNA expression of IL-1β. In this chapter, over-expression of Nurr1 isoforms in adult microglia decreased IL-1β and iNOS mRNA expression upon ATP exposure. Data showed that IL-10 mRNA expression was higher only in TINUR-overexpressed adult microglial cells. Covalent attachment of SUMO to transcription factors including Nurr1, has been suggested to be crucial to promote the recruitment of transcriptional co-repressors to inflammatory promoters (Yang and Sharrocks, 2004). Since the SUMOylation sites are located in the LBD/AF2 domain (Saijo et al., 2009b). Nurr1a isoform was not able to trans-activate IL-10 expression, but it can still trans-repress which suggests that SUMOylation may not be required for trans-repression by Nurr1. Recently, Sáez et al.

(2018) reported that CoREST are SUMOylated, and they revealed that PIASγ increases the stability of CoREST proteins and facilitates their SUMOylation by SUMO-2.

In this study, data showed that over-expression of Nurr1 isoforms in LPS-induced neonatal and adult microglia had no effect on both pro- and anti-inflammatory cytokines. De Miranda et al. (2015) reported different findings that Nurr1 inhibited LPS-induced expression of NF-kB regulated genes in BV-2, but they treated the cells with Nurr1 activator 1,1-Bis(3'-indolyl)-1-(p-Chlorophenyl) Methane. The difference between the current study finding and the De Miranda study is the cell type and the use of overexpression versus Nurr1 activator. Another study showed that, in monocytic cells, adenosine modulates the expression of Nurr1 through A2a receptor to limit NF-$_k$B mediated effects (Crean et al., 2015). Collectively, it is worthy to investigate the pharmacologic stimulation of Nurr1-mediated trans-repression by activators in primary microglia model to decrease expression of NF-$_k$B-regulated inflammatory genes.

It was shown in Chapter 4, that neonatal microglia displayed anti-inflammatory phenotype which were characterized by increased mRNA expression levels of IL-10. It should be noted that, these data indicated that the primary function of microglia during the early postnatal period may be to participate in building the CNS (Fitzgerald and McKelvey, 2016). In other words, the anti-inflammatory phenotype to PAMPs and DAMPs in neonatal microglia may be the indirect outcome of the requirements for normal postnatal development in the CNS (Bremner and Fitzgerald, 2008, Koch et

al., 2012). However, it was of interest to know whether Nurr1 isoforms would have a role to maintain the anti-inflammatory response of microglia in neonates.

Data in this chapter show that over-expression of TINUR isoform in neonatal microglia induced IL-10 mRNA expression after ATP simulation and this again is a confirmation of the findings obtained from reporter gene assay. Interestingly, over-expression of TINUR in ATP-stimulated neonatal microglia brought the iNOS expression down to the level of control group. This clearly indicates that overexpression of Nurr1 in microglia may play a crucial role in protecting microglia from inflammation. Experiments conducted in our group employing sequential transfer of cell culture media from neonatal and adult microglia to astrocytes show that astrocyte can amplify the microglia-derived factors in the release of neuroprotective and neurotoxic mediators, including Nurr1 and IL-1β respectively. Moreover, decrease of Nurr1 expression in microglia and astrocyte led to increase the release of neurotoxic mediators in response to inflammatory stimuli (Saijo et al., 2009a). Moreover, in order to obtain make-up of functional neuronal circuits particularly in early life, it is needed to inhibit the release of neurotoxins mediators by both microglial cells and astrocytes (Brundin et al., 2008).

Furthermore, over-expression of Nurr1 variants in neonatal microglia has no effects on the inflammatory response after stimulation with both HMGB1. A possible explanation for these observations is that HMGB1 mainly activate RAGE receptors but it is also a TLR4 ligand. Activation of TLR4 results in activation of the MAPK cascades, leading to activation of NF-$_k$B (Pålsson-McDermott and O'neill, 2004).

To conclude, the data in this Chapter provides a novel insight into the anti-inflammatory effect of TINUR isoform in microglia. The work explores how over-expression of this isoform can exert anti-inflammatory effects by down-regulating the mRNA expression of IL-1β and iNOS, and enhancing the mRNA expression of IL-10 in both neonatal and adult microglia particularly after ATP stimulation. Nurr1 appears to be a key transcription factor that contribute to the balance of different microglial responses, when the Nurr1 is overexpressed in the microglia, the pathologic microglia with pro-inflammatory phenotype may be converted into microglia with an anti-inflammatory phenotype that secretes neurotrophic factors protecting the astrocytes and neurons.

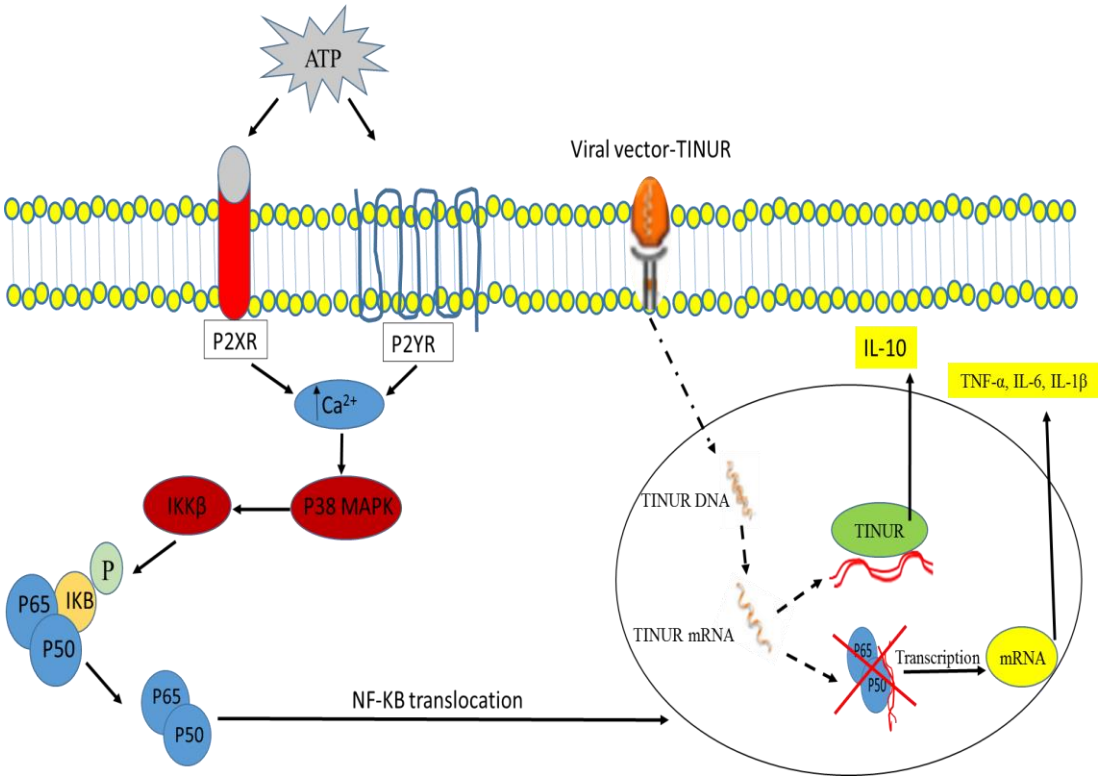


Figure 5.22. Proposed mechanism for TINUR (Nurr1 isoform) mediated anti-inflammatory effects in microglia after ATP activation. Activated microglia release both pro- and anti-inflammatory cytokines. In this model of *in vitro* ATP activation, microglia become activated and increase inflammatory cytokines expressions. Overexpression of TINUR using lentiviral methods causes trans-repression of pro-inflammatory genes including IL-1β and iNOS, and trans-activation of anti-inflammatory gene IL-10.

6 Chapter six: General discussion

6.1 Summary of findings

The introduction of this thesis described microglia as having a multifaceted nature. There is a significant level of uncertainty as to how microglial activation is regulated during the postnatal period. Although several mechanisms have already been identified, there are other factors that could potentially have a role. The main aim of the studies described in chapter 3 was to investigate the expression of endocannabinoids signalling system during development in human brain tissue. In this Chapter, it was shown age-related alterations in the expression of key enzymes and receptors that are involved in endocannabinoid signalling system. The data described in chapter 4 focused on the functional phenotype of neonatal and adult microglia isolated from rat brain and spinal cord. In addition, the response of different populations of microglia (brain vs spinal cord and neonatal vs adult) to stimulation with known microglial stimulating ligands including PAMPs and DAMPs was compared. The findings of this chapter have established procedures to generate a pure single cell population of neonatal and adult microglia from rat spinal cord and brain. The application of this preparation is to reveal the regional microglia heterogeneity in both neonate and adult rats. Neonatal and adult Microglia that were exposed to PAMPs and DAMPs responded differently towards anti-inflammatory and pro-inflammatory phenotypes, respectively. Neonatal microglia did not show an increase in the expression of pro-inflammatory IL-1β contrary to adult cells upon ATP and HMGB1 exposure. However, expression of the anti-inflammatory IL-10 gene was significantly increased in neonatal cells compared to adult cells. These findings provided evidence for functional changes in the central immune response during early life showing a shift from an anti-inflammatory response in early life to a pro-inflammatory response in adulthood. The question was then to find a method of converting adult microglia from

pro-inflammatory phenotype to anti-inflammatory phenotype. The aims in chapter 5 addressed this question by studying the neuroprotective and anti-inflammatory roles of transcription factor Nurr1 isoforms in stimulated microglia. Nurr1 trans-repression pathway in microglia and astrocytes protects dopaminergic neurons from inflammation-induced death (Saijo et al., 2009a). The findings of this study have shown that TINUR overexpression exhibited anti-inflammatory effects by decreasing the pro-inflammatory cytokines expression levels in ATP-induced adult microglia. The results further revealed that different isoforms of Nurr1 were expressed in neonatal and adult microglia. All of the isoforms were able to attenuate the expression of inflammatory mediators in ATP-activated neonatal and adult microglia. However, only TINUR was able to induce the expression of anti-inflammatory cytokine in ATP-stimulated neonatal and adult microglia.

6.2 Overall significance of findings

6.2.1 The expression of endocannabinoid system in postnatal maturation of nociceptive processing in human brain

The cannabinoid signalling system is essential for mediating the maturation of pain signalling system during CNS development. This thesis has outlined that the endocannabinoid system undergoes age-related changes in the midbrain and cerebellum. The data obtained from this thesis have shown that infants and adults have remarkable differences in their abilities for endocannabinoid synthesis, degradation, and receptors expressions. While the specific circumstances surrounding availability post-mortem samples was a major limitation for further use of human brain materials. The findings of this study have revealed that endocannabinoids receptor CB_1 mRNA expression in human midbrain and cerebellum significantly decreased in adulthood,

and these findings suggest a robust regulation of neurotransmission by CB_1 in early life. Neonatal NAPE-PLD mRNA expression levels increased during infancy by tenfold and threefold in midbrain and cerebellum, respectively. These findings are in concordance with previous observations that showed an increase in cortical NAPE-PLD expression from postnatal day 14 to 56 in rat brain (Morishita et al., 2005, Kwok et al., 2017). Overall, these data suggests that the capacity for anandamide synthesis process by the NAPE-PLD pathway may be more efficient in maturity than in early life in both rat and human brains.

The findings of this study may reveal important insights in the identification of pathophysiological mechanisms in developmental disorders that are related to glial migration, axon elongation and synaptogenesis (Saez et al., 2014, Guy et al., 2015). For example, previous studies have shown that children prenatally exposed to marijuana showed a significant change in the regulation of the differentiation and migration of glutamatergic and GABAergic cortical neurons during development. Those alterations might result in an improper association of neuronal networks that could in turn cause a cognitive and neurobehavioral dysfunction in adulthood (Mereu et al., 2003, Antonelli et al., 2004). Importantly, the adolescence period is the most probable time to start using cannabis, the findings of this study highlight the need to investigate how exposure to exogenous cannabis compounds during this period influence the endocannabinoid system expression.

6.2.2 Age- and location-related changes were observed in inflammatory cytokines expression, nitric oxide production and phagocytic activity

CNS has unique and important immunological component. There is mounting evidence to indicate that microglia achieve many important functions that are essential to maintain homeostasis. The normal microglial functions include its ability to detect deviations in the microenvironment through the expression of a range of functional immunological factors that help them to respond properly to different environmental stimuli. The data of the current study have shown that microglia functions were significantly affected by microenvironment and accumulative properties of ageing. At the baseline, the inflammatory cytokines were significantly expressed in microglia, but there were differences in their expression profiles in brain and spinal microglia. The higher basal inflammatory cytokine levels in the spinal microglia may contribute to the activation of these cells, particularly at adulthood, leading to the release of a battery of pro-inflammatory mediators that disrupt normal maintenance functions resulting in neurodegenerative diseases. As demonstrated in chapter 4, the phagocytic activity in adult microglia was decreased compared to neonatal cells at basal line. These decreases in debris clearance have been associated with the pathogenesis of age-related disorders such as AD (Sokolowski and Mandell, 2011). The reduction in substrate uptake and proteolytic capacity is usually associated with ageing, which leads to a decrease in breakdown efficacy of β-amyloid (Li, 2013). β-amyloid triggers microglial release of pro-inflammatory mediators that suppress phagocytosis (Hanisch and Kettenmann, 2007).

6.2.3 A natural alteration in developmental role for anti-inflammatory activity of microglia in the CNS

Emerging data suggest that neuropathic pain can appear later in life following early nerve injury due to changes in neuro-immune cells in CNS throughout development (McKelvey et al., 2015). In early life, microglial cells activity within the spinal cord helps to support the neural circuits growing in normal environment. As described in Chapter 1, microglia are crucial for synaptic pruning and neuronal apoptosis during CNS development (Paolicelli et al., 2011, Schafer and Stevens, 2015). The data of the current study have demonstrated that microglia undergo remarkable alterations in both morphology and phagocytic activity through the postnatal period, showing an activated morphology and high phagocytic activity following PAMPs and DAMPs stimulations in neonatal microglia, in contrast to highly motile processes and low phagocytic activity in adult cells. Neonatal microglia also showed higher mRNA transcript levels of anti-inflammatory cytokine IL-10 compared to the adult cells upon exposure to PAMPs and DAMPs. This suggests that their activities in the early postnatal period may be different from those during adulthood. In addition, the density of microglia in the postnatal brain is two folds higher than in the adult mouse (Nikodemova et al., 2015), demonstrating that increased apoptosis and decreased proliferation rate may have a crucial role in controlling microglial number and activity at adulthood.

The findings of this study indicated that the immune response in neonatal microglia is primarily anti-inflammatory which is consistent with the recently observed anti-inflammatory response to nerve injury in early life (PrabhuDas et al., 2011, Elahi et al., 2013, McKelvey et al., 2015). In addition, the microglial cells undergo

developmental changes with age from an anti-inflammatory phenotype at early life shifted to pro-inflammatory responses in adult microglia in both brain and spinal cord. A possible explanation for the anti-inflammatory response that was observed in neonatal microglia in response to PAMPs and DAMPs may be to avoid harmful inflammation following the move from a sterile in utero environment to exposure to microbes post-natally (Maynard et al., 2012). Microglia during postnatal development have a crucial role that is related to the significant alterations in the connectivity of the CNS over the postnatal period including removing cellular debris via phagocytosis, controlling the size of dendritic spines and establishing proper connections with synapses (Arnoux et al., 2014). Tremblay et al. (2010) reported that microglia actively engulf synapses and control the number of synapses in response to changes in cortical vision sensory in juvenile animals. These findings were confirmed by microglial depletion studies. For instance, administration of the microglial inhibitor minocycline or depletion of microglial fractalkine receptor leads to abnormal maturation of synaptic circuits (Paolicelli et al., 2011, Schafer et al., 2012). Collectively, the anti-inflammatory response of microglial cells to PAMPs and DAMPs in early life may be essential for normal postnatal development through CNS.

6.2.4 Anti-inflammatory effects of Nurr1 by converting the phenotype of activated-microglia

The idea of a protective autoimmunity of the CNS has been established and has been used to improve immunomodulatory therapies for neurodegenerative disorders, thereby increasing the protective and regenerative features of the immune system for neuroprotection in CNS diseases (Butovsky et al., 2006, Graber and Dhib-Jalbut, 2009). Furthermore, microglia are considered a promising target to design new

therapeutic approaches for neurodegenerative disorders such as AD, PD and multiple sclerosis (Asheuer et al., 2004, Guo et al., 2004, Liu et al., 2017).

The findings in chapter 5 revealed that the overexpression of Nurr1 isoforms particularly the TINUR in microglia using lentivirus methods exhibited anti-inflammatory effects by converting the activated-microglia towards anti-inflammatory phenotype. The main significance for these findings is that the Nurr1 isoforms, are endogenous transcription factors that are expressed in glial cells and dopaminergic neurons in physiological conditions, thus strengthening the intrinsic microglial anti-inflammatory phenotype to promote the survival of these neurons and protect them against toxic factors. However, the levels of Nurr1 decreased during ageing and degeneration (Decressac et al., 2013). In addition to the anti-inflammatory role in the microglia, the overexpression of Nurr1 shifts activated microglia toward an anti-inflammatory phenotype by reducing the levels of IL-1β as well as increasing the expression of IL-10.

6.3 Future work

In order to address whether there is association between SUMOylated Nurr1 and its trans-repression and trans-activation activities on the inflammatory genes, a number or essential gaps need to be filled.

The results of this thesis has described a novel way of converting microglial phenotype from pro-inflammatory to anti-inflammatory phenotype by increasing the expression of a promising nuclear receptor Nurr1. Further studies with *in vivo* models such as

neuropathic pain and AD models may help to provide more detailed and reliable evidence to reveal regulation of microglial Nurr1.

This work provides no evidence of the cellular distribution of Nurr1 in microglia. Further works to investigate the mechanism of regulation of nuclear/ cytoplasm shuttling of Nurr1 to explain how the inflammation participates in Nurr1 transcriptional activity will be helpful. In addition, Future studies will be required to determine whether inflammatory stimuli affect the nuclear localization of Nurr1 may participate in loss of dopaminergic phenotype in pathologies such as PD.

Data from this thesis show that the phagocytic activity decreased in adult microglia compared with neonatal cells. Further studies would be helpful to determine the therapeutic efficiency of targeting phagocytic pathways in microglia in adulthood.

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