Neuregulin's role in regulating the anti-inflammatory pathway

by

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Author's declaration

I herby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Inflammation can be up-regulated by microglia and macrophages through the release of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α). Excess production of TNF-α can lead to a variety of diseases and even tissue necrosis. Recently, the expression of alpha seven acetylcholine receptors (α7AChR) by microglia have been shown to decrease the amount of TNF-α released. This anti-inflammatory pathway has been studied extensively where researchers are able to reduce TNF-α concentration through α7AChR expression and increases in the concentration of its ligand. I have shown that Neuregulin is able to increase the expression of α7AChR in microglia and macrophages.

Using three immortalized cell lines, BV-2, EOC-20 and RAW 264.7, and primary microglial cells harvest from mice I investigated the role that neuregulin plays in the anti-inflammatory process. Neuregulin signals through the ErbB receptors, a family of tyrosine kinase receptors, to facilitate the effects on ACh expression. My results show that ErbB4 is expressed in BV-2, EOC-20 and RAW 264.7 cell lines while ErbB2-4 receptors are expressed in primary microglia. As well, I was able to show that ErbB4 became phosphorylated upon binding to NRG in immortalized cell lines.

Using an Enzyme Linked Immunsorbent Assay to analyze TNF- α concentration in microglia and macrophages, I was able to demonstrate that increased levels of α7AChRs did not result in a reduction in TNF-α concentration. These results showed that NRG is able to increase α7AChRs in microglia and macrophages after the phosphorylation of the ErbB4 receptors. As well, this increase in α7AChR does not relate

to a reduction in TNF-α, thus under these experimental conditions does not have an effect on the anti-inflammatory pathway.

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Table of Contents

List of Figures

List of Tables

Abbreviations

α7AChR	Alpha Seven Acetylcholine Receptor
AD	Alzheimer's disease
APC	Antigen Presenting Cells
ARIA	Acetylcholine Receptor Inducing Activity (synonym: neuregulin)
BSA	Bovine Serum Albumin
BV-2	Mouse microglial cell line
C2C12	Mouse myoblast cell line
CNS	Central Nervous System
CSF-1	Colony Stimulating Factor-1
DIC	Differential Interference Contrast
DiI-Ac-LDL	Acetylated Low Density Lipoprotein, labeled with 1,1'-dioctadecyl – 3,3,3',3'-tetramethyl-indocarbocyanine perchlorate
DIV	Days *in vitro*
DMEM	Dulbecco's Modified Eagle's Medium
D-PBS	Dulbecco's Phosphate Buffer Saline
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunosorbent Assay
EMEM	Eagle's Minimum Essential Medium
EOC-20	Mouse microglial cell line
FBS	Fetal Bovine Serum
GGF	Glial Growth Factor (synonym: neuregulin)

HBSS	Hank's Balanced Salt Solution
HeLa S3	Human cervical epithelia line
HRG	Heregulin (synonym: neuregulin)
HRP	Horse Radish Peroxidase
IL-1	Interlukin-1
IL-12	Interlukin-12
LADMAC	Mouse lymphoblast cell line
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
NDF	Neu Differentiation Factor (synonym: neuregulin)
NF-κB	Nuclear Factor kappa B
NMJ	Neuromuscular Junction
NRG	Neuregulin
PAMPs	Pathogen-Associated Molecules
PBS	Phosphate Buffer Saline
PNS	Peripheral Nervous System
RAW 264.7	Mouse macrophage cell line
SDS	Sodium dodecyl sulfate
SMDF	Sensorimotor-dervided factor (synonym: neuregulin)
TLR	Toll like receptor
TNF- α	Tumour necrosis factor alpha

Chapter 1 Introduction

1.1 The central nervous system and inflammation

The central nervous system (CNS) is an immune-privileged site that is inaccessible to cells of the systemic immune system due to the blood brain barrier. The blood brain barrier is composed of endothelial cells linked together by tight junctions and exclude cells from circulating blood. As a result of this physical separation, the CNS has its own immune system composed of microglial cells. These resident immune cells have the capability to degrade foreign invaders and recruit others for help, principally by increasing the permeability of the blood brain barrier and allowing for cells of the systemic immune system to enter into the CNS.

The immune response normally progresses from an innate to an adaptive response. In the initial steps of an innate response inflammation is a normal physiological process that occurs throughout the body where cells of the immune system are recruited to areas of infection, injury and trauma (Pavlov et al., 2003). While this is normally a beneficial process where the cells of the immune system aid in the clearing of damaged tissue, debris and pathogens, it can be detrimental especially when this type of response occurs in the central nervous system (Pavlov et al., 2003). When a local inflammatory response is uncontrolled, systematic inflammation may ensue resulting in shock, possible organ failure, swelling in the brain and spinal cord and even death (Pavlov et al., 2003). Innate deregulation can also lead to a variety of auto-inflammatory diseases including arthritis, asthma, Graves disease and Crones disease (Targan et al., 1997; Salvi et al., 2000). In

many of these diseases there is an over expression of Tumour Necrosis Factor alpha (TNF-α), a pro-inflammatory cytokine (Targan et al., 1997; Salvi et al., 2000; Chabot et al., 1997).

1.2 The innate immune response

The innate response is the initial response to a foreign invader or injury. It is often initiated by the presence of pathogen-associated molecules (PAMPs), molecules broadly shared by pathogens but distinguishable from host molecule. PAMPs are recognized by cells of the immune system, in particular antigen presenting cells (APC). APC are a group of cells that are capable of internalizing an antigen, degrading it and presenting it on their major histocompatibility complex (MHC) class two receptors to T lymphocytes (Nguyen et al., 2002). In the CNS, microglia are able to perform as APCs upon encountering a pathogen. Upon invasion of the foreign invader into the CNS, the PAMPs on the intruder are recognized by Toll-Like Receptors (TLR). Depending on the type of PAMP present, a different TLR will be activated for instance, TLR-4 is activated in response to Lipopolysaccharide (LPS) found on gram-negative bacteria (Olson and Miller, 2004; Nguyen et al., 2002). PAMPs binding to TLRs induce APC activation, which leads to secretion of a variety of chemokines and cytokines responsible for recruiting peripheral macrophages and T cells. The activation of microglial cells leads to the induction of the nuclear factor kappa-beta (NF-κβ) pathway where it's activation leads to a transcription of a variety of pro-inflammatory cytokines including TNF-α (Olson and Miller, 2004; Wang et al., 2003) (Figure 1.1).

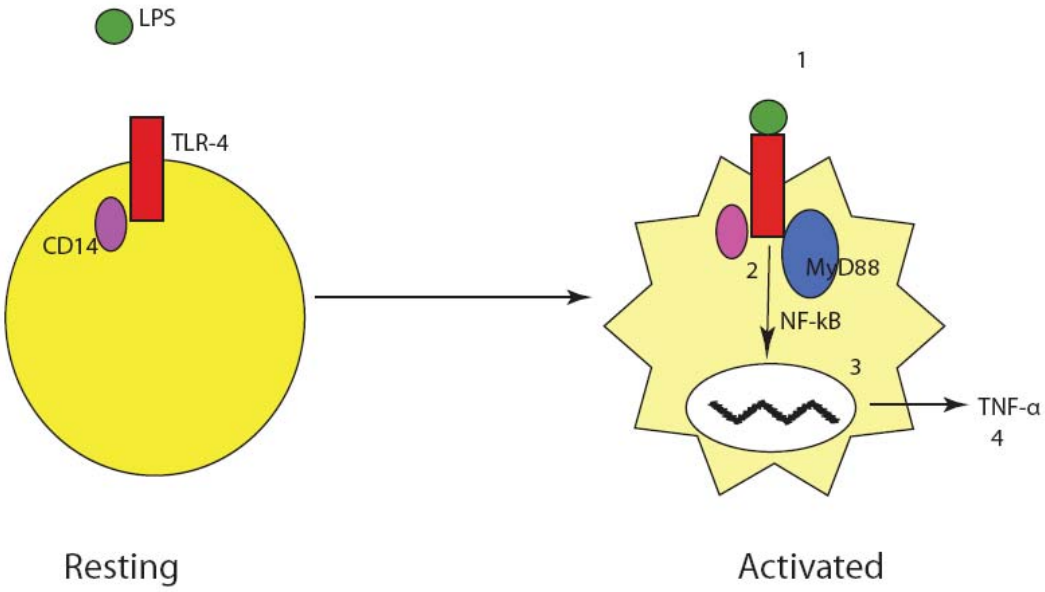


Figure 1.1: Microglial activation and release of TNF-α. Microglia reside in a resting state (left) until they encounter a foreign invader or are activated by cytokines (right). LPS on gram-negative bacteria (or other PAMPs) stimulate Toll-like receptors (TLRs) on the surface of microglia activating the cell (1). TLR-4 in association with CD14 recognizes LPS and through their interaction with the adaptor protein MyD88 (2) they stimulate the NFκB signaling pathway and trigger TNF-α transcription (3). TNF-α induces neutrophil proliferation, activates more microglia and provides a positive feedback loop to increase NFκB and subsequently producing more TNF-α (4).

Inflammation occurs in conjunction with the innate response and is initiated by similar mechanisms. Inflammation begins with contraction of smooth muscle around the large blood vessels to increase the flow of blood through capillaries at the site of infection. Next extravasation occurs, allowing the once tight junctions between endothelial cells of the blood brain barrier to become permeable to cells of the immune system (Figure 1.2) (Stamatovic et al., 2008). This process culminates with diapedesis where cytokines and complement molecules act as adhesion molecules allowing circulating leukocytes to attach to the surface of endothelial cells, flatten and squeeze through the intervening spaces (Sage and Carman, 2009).

Inflammation is maintained through activated macrophages through the release of different inflammatory cytokines such as TNF-α and IL-1 after their encounter with the foreign invader. TNF-α and IL-1 bind to receptors on endothelial cells that are required for maintaining extravasation thus continuing the inflammatory response (Secher et al., 2009).

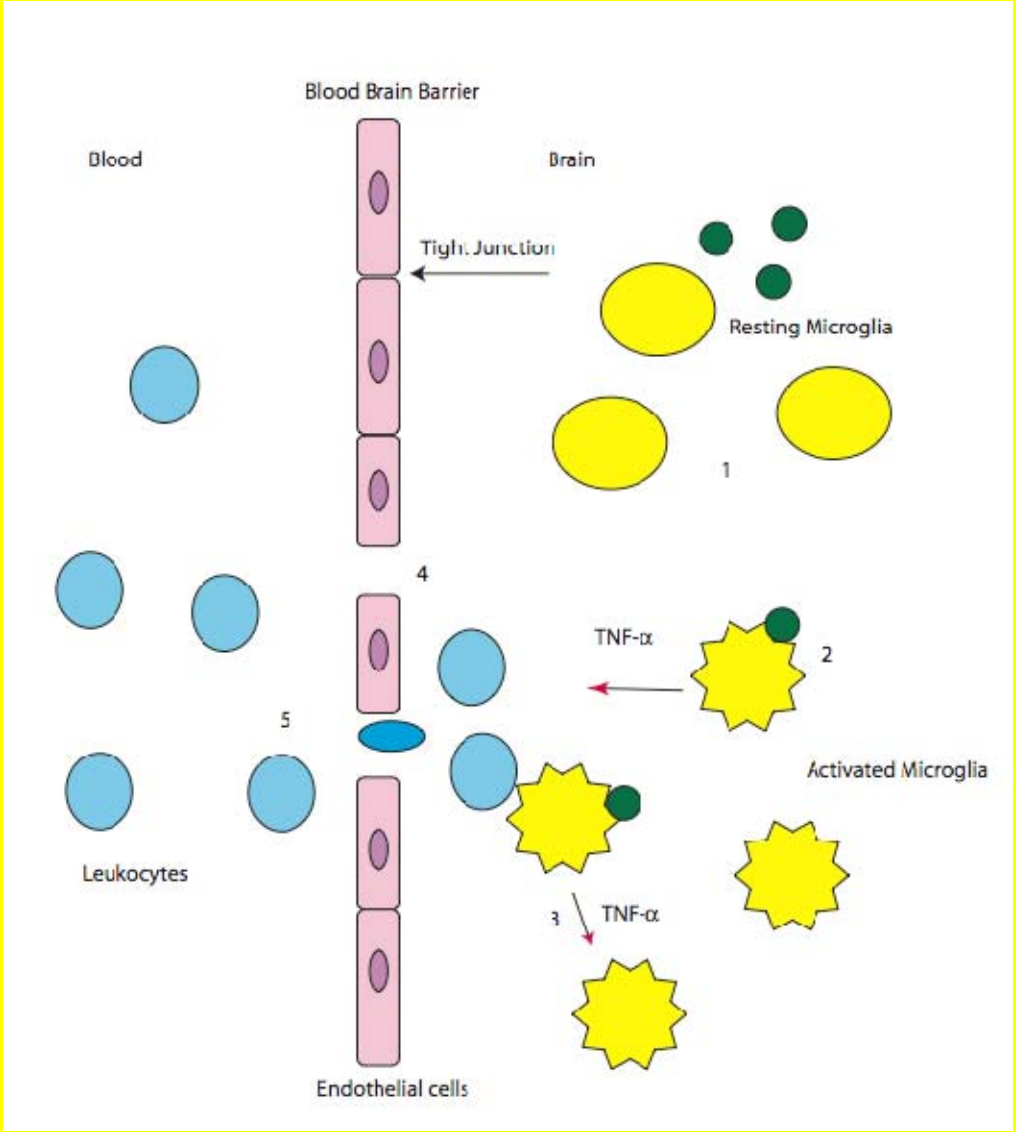


Figure 1.2: Inflammation. Inflammation begins with recognition of a foreign invader or damage (1). In the case of CNS, most foreign invaders are recognized by microglia. Upon interaction microglia become activated (2) and secrete TNF-α to activate surrounding microglial cells (3) and to initiate vasodilation. The tight junctions of endothelial cells that create the blood brain barrier loosen as endothelial cells constrict in response to a foreign invader (4). This contraction allows for leukocytes of the systemic immune system to squeeze through the barrier and enter into the CNS (5). Cells of the systemic immune system are attracted to the CNS through the release of TNF-α.

1.3 Microglia: The resident CNS immune cell

The main cellular mediators regulating inflammation in the brain are microglia. Derived from myeloid cells of the periphery, these resident immune cells comprise around 12% of the total cell population in the brain (Block et al., 2007). Upon activation by foreign invaders, microglia change from a predominantly resting state that is characterized by an amoeboid morphology to an active state. This activation allows for better recognition of pathogens, signaling to other immune cells and induces the transcription of cytokines. Microglia are key mediators of innate immunity because they secrete a variety of cytokines, are active in the phagocytosis of debris and pathogens and are involved in the recruitment of other immune cells and activation of the adaptive immune response (Wang et al., 2003; Pavlov et al., 2003; Streit et al., 2004; Raivich and Banati, 2004; Matsunaga et al., 2001).

Microglia have the ability to be both beneficial and harmful to the CNS depending on the level of stimulation they receive and the cytokines subsequently released. Microglia can be quite destructive to the CNS when there is an overproduction of superoxides, nitrogen oxide and TNF-α; all molecules responsible for tissue damage and dysregulation of inflammation. Microgliosis, a state in which microglia become activated, can lead to these increases in inflammatory molecules and are correlated to a variety of neurodegenerative diseases such as Alzheimer's, Parkinson's, HIV dementia, Multiple Sclerosis and Huntington's disease (Streit et al., 2004; Block et al., 2007; Nguyen et al., 2002; Hensley et al., 2003).

1.4 Microglia and the anti-inflammatory pathway

Research has shown that in the PNS, macrophages can regulate the systemic inflammatory response via α7AChRs (Wang et al., 2003; Jonge and Ulloa, 2007). α7AChR expression on macrophages has been shown to decrease the amount of TNF-α released in response to an assault in the PNS (Wang et al., 2003) (Figure 1.3). The reduction in TNF-α expression results in a decrease in the inflammatory response. Being able to mediate the inflammatory response could result in potential therapies for diseases where inflammation is responsible for the majority of damage.

Microglia have also been shown to be able to participate in this anti-inflammatory control through the expression of α7AChR (Shytle et al., 2004). It is not surprising that α7AChRs are present in both microglia and macrophages since they are derived from the same progenitor cell. Finding the pathway responsible for regulating the transcription of α7AChR in microglia will possibly lead to a mechanism for regulating the inflammatory response.

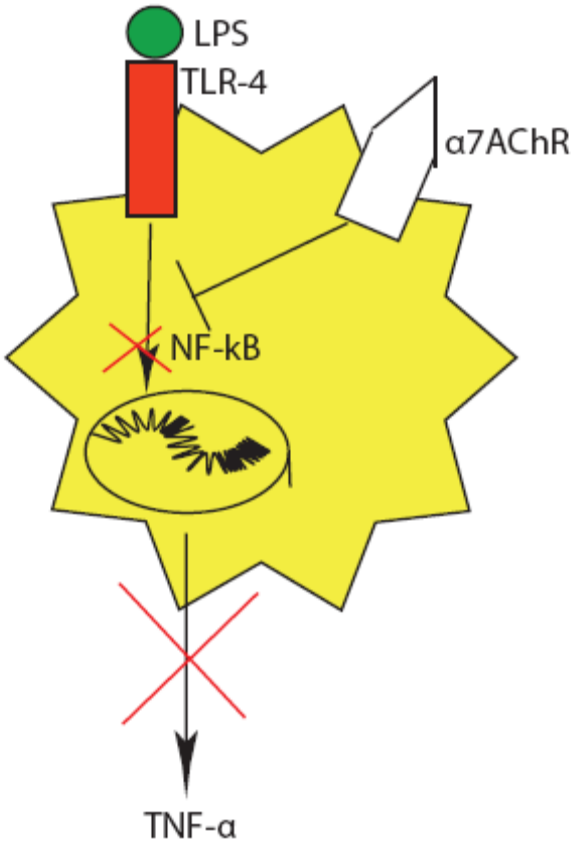


Figure 1.3: Microglia and the anti-inflammatory pathway. Acetylcholine is an important neurotransmitter in the brain that signals through nicotinic (ligand-gated ion channels) or muscarinic (G-protein coupled receptors). The binding of a specific subset of nicotinic acetylcholine receptors, α7AChR, are able to regulate inflammation through a mechanism called "nicotinic anti-inflammatory pathway." In this pathway, inflammation is regulated through the binding of nicotine or acetylcholine to α7AChR, which inhibits the production of pro-inflammatory cytokines such as TNF-α.

1.5 Neuregulin and the ErbBs

Neuregulins are a family of glycoproteins that have diverse roles in both CNS and PNS (Falls, 2003; Dimayuga et al., 2002; Zhenfeng et al., 2004). Historically, the NRG family has included names such as: acetylcholine receptor-inducing activity (ARIA), neu differentiation factor (NDF), glial growth factor (GGF), heregulin (HRG) and sensorimotor-derived factor (SMDF). All of these names were originally derived from their described functions within the CNS and PNS but later were reconciled under the neuregulin nomenclature based on similarities in structure. NRG is now classified based on which of the four NRG genes it comes from, NRG1-4. The functional differences of each NRG glycoprotein arise due to alternative splicing and differential promoter usage within the neuregulin gene but are still classified according to its origin (Dimayuga et al., 2003).

NRG-1 and -2 share the highest sequence homology with each other, while NRG-3 and 4 are distantly related to NRG-1 (Buananno and Fischbach, 2001; Falls 2003). NRG-1 was the first gene to be cloned, is the most widely expressed and the most extensively researched. NRG-1 is expressed in neurons, glial cells, heart, muscle, liver, stomach, lung, kidney, spleen and skin where it has been implicated in various biological processes at the neuromuscular junction, including organizing the postsynaptic membrane, the regulation of nicotinic acetylcholine receptor (AChR) transcription and the induction of muscle spindle formation (Buananno and Fischbach, 2001; Falls, 2003; Rimer, 2003; Jacobson et al., 2004). NRG-1 is used in this thesis because of its ability to regulate AChR transcription in muscle cells. Of the remaining NRGs, NRG-2 is expressed in heart and CNS, NRG-3 is found only in CNS and NRG-4 is found within the PNS, pancreas and skeletal muscle

(Buonanno and Fischbach, 2001). Ultimately the mechanism of NRG action depends on its binding to a heterogenous family of receptor tyrosine kinases related to the EGF receptor.

NRG binds and signals through the ErbB receptors, a family of tyrosine kinase receptors consisting of four members, ErbB1-4 (Yarden and Sliwkowski, 2001). NRG can bind directly to two members of this family; ErbB3 and ErbB4. Once bound, these receptors can form homodimers, in the case of ErbB4, or recruit ErbB1 or ErbB2 to form heterodimers (Buananno and Fischbach, 2001). Binding of NRG to the ErbBs is via an epidermal growth factor (EGF) domain common to all neuregulins (Buananno and Fischbach, 2001). Binding initiates phosphorylation leading to a variety of signaling cascades that can result in apoptosis, cell migration, growth, adhesion or differentiation depending on the ErbB receptor dimer that NRG binds (Yarden and Sliwkowski, 2001) (Figure 1.4).

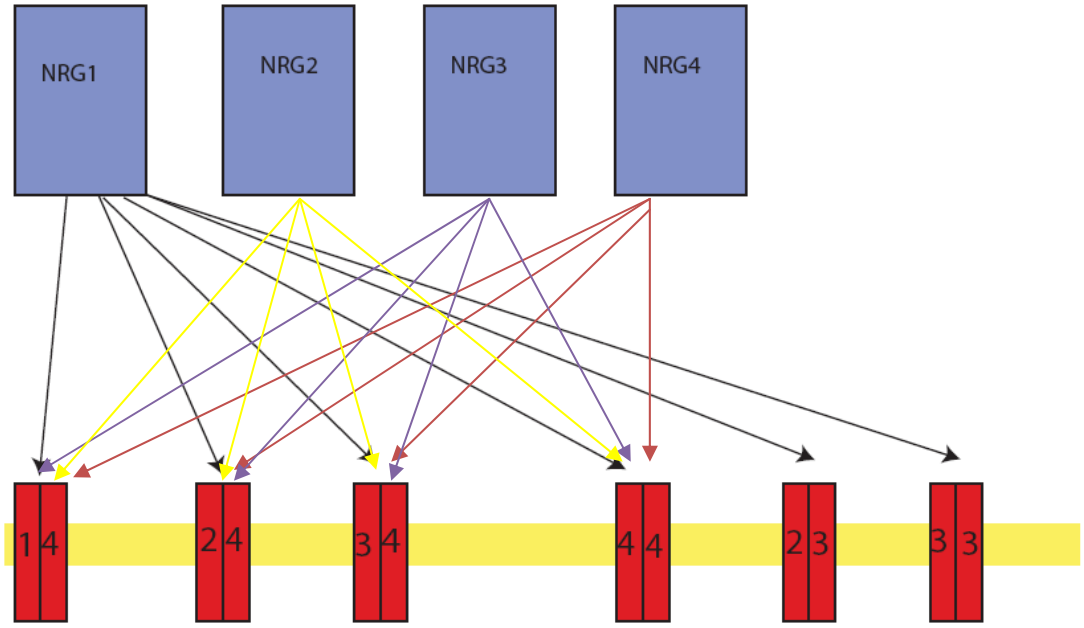


Figure 1.4: Neuregulins and the ErbB receptors. There are a variety of ligands that can bind to the ErbB receptors (red), including the NRGs (blue). Depending on the ligand, along with different homodimer and heterodimer receptor combinations, will signal a variety of signaling ending with apoptosis, migration, growth, adhesion and differentiation of the cell. NRG1 can bind to ErbB3 and ErbB4 in any combination while NRG2-4 can only bind to ErbB4 receptors (Figure adapted from Yarden and Sliwkowski, 2001).

ErbB2-4 are three ErbB receptors that are investigated in this thesis and are critical for NRG-1 signaling. Of these receptors, ErbB2 currently has no known ligand but is recruited to form heterodimers with the other ErbB receptors (Yarden and Sliwkowski, 2001; Feroz et al., 2002). ErbB3 is found expressed in many different types of neural cells and epithelial cells. Devoid of intrinsic kinase activity it forms heterodimers with the other ErbB receptors to initiate signaling. The combination of ErbB2-ErbB3 results in a heterodimer that binds effectively to NRG-1 and is a potent stimulator of acetylcholine receptor transcription.

In the CNS, ErbB3 is found expressed in oligodendrocytes, the glial cell responsible for myelinating axons of neurons (Feroz et al., 2002; Yarden and Slwkowski, 2001; Cannella et al., 1999). ErbB4 is also found in the central nervous system and is able to bind to all four neuregulin isoforms. ErbB4 expression is seen throughout the neural cell population and is upregulated in response to injury (Erlich et al., 2000). ErbB4 is unique in comparison to the other three receptors where it has four different isoforms generated through mRNA splicing (Carpenter, 2003). Two of the isoforms (JM-a and JM-b) are membranous while the other two are cytoplasmic (CYT-1 and CYT-2) (Carpenter, 2003; Sharif et al., 2009) but all four isoforms are expressed within the central nervous system (Junttila et al., 2000).

1.6 Neuregulin and regulating expression of acetylcholine receptors

Prior to implementing the presently used neuregulin nomenclature NRG-1, one isoform was known by the name ARIA for acetylcholine receptor-inducing activity (Fischbach and Rosen, 1997). ARIA was discovered due to its ability to increase AChR gene expression at the neuromuscular junction (NMJ) (Fischbach and Rosen, 1997). ARIA now NRG-1, is released from the developing motor neuron terminal, activating its receptor (ErbB2-ErbB3) on the post synapse thereby inducing AChR gene expression (Pun et al., 1997).

At the neuromuscular junction, neuregulin is involved with the induction of a signal transduction cascade ending with the activation of AChR subunit gene transcription (Falls, 2003). The cascade starts with the binding of NRG-1 to the ErbB2-ErbB3 heterodimer complex. This binding induces the phosphorylation of the receptors and the subsequent activation of the Ras/Raf/ERK MAPK pathway. The end result of this signaling event is the transcription of AChR in muscle cells (Figure 1.5). If NRG is able to regulate AChR in muscle cells, then it is possible that a similar regulation of AChR can occur in microglial cells.

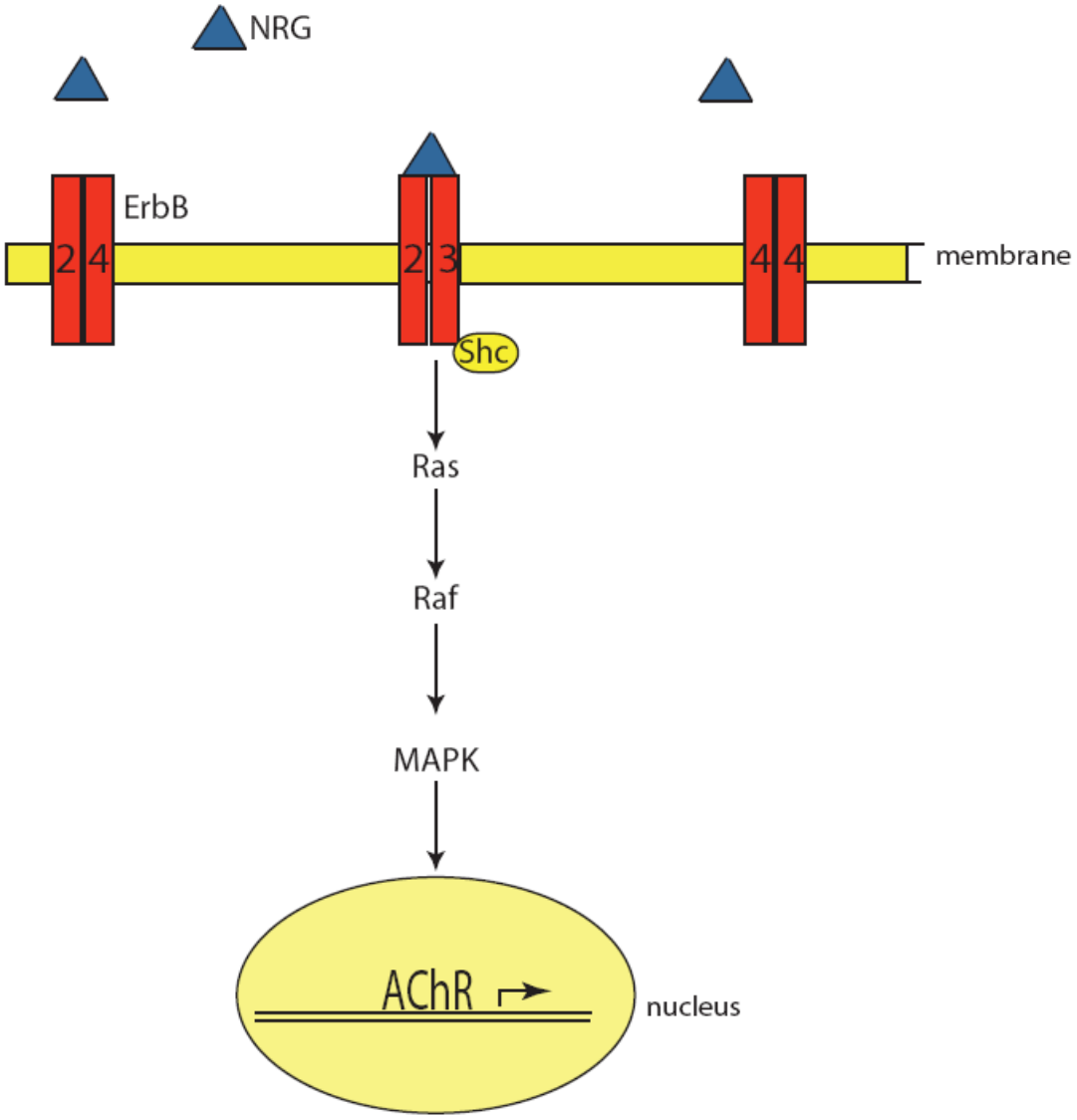


Figure 1.5: Neuregulin induces acetylcholine receptor transcription at the NMJ. In muscle cells, activation of ErbB tyrosine phosphorylation by NRG-1 causes an association with the adaptor protein Shc. This complex stimulates the Ras/Raf/ERK mitogen-activated protein kinase (MAPK) pathway. Briefly, this signaling pathway ends with the transcriptional upregulation of AChR at the neuromuscular junction. Neuregulin is identified as a triangle and the boxes labeled with 2, 3, and 4 represent the ErbB receptors.

1.7 Experimental objectives of this study

This study aims to investigate the possible effects NRG may have on microglia. In particular I am interested in the possible up-regulation of α7AChR expression and NRG's potential to regulate inflammatory responses. I hypothesize that the addition of NRG may increase the expression of α7AChR.

Without ErbB expression, NRG would be unable to bind microglia to effectively mediate changes in gene expression or ultimately inflammation. Due to inconsistencies in the literature (Erlich et al., 2000; Dimayuga et al., 2002; Gerecke et al., 2001; Cannella et al., 1999; Chaudhury et al., 2003) my initial experiments examined ErbB receptor expression in primary microglia and several microglial cells lines via immunoblot. Immunoblots of α7AChR expression were compared in untreated, NRG treated, NRG and LPS treated and LPS treated cells to determine these molecules effects on α7AChR expression.

Lastly, to observe NRG's affect on the inflammatory response TNF-α was quantified using an ELISA. Again, samples were treated with NRG, LPS and NRG and LPS alone. Since TNF-α is a pro-inflammatory cytokine, an increase in expression typically results in a stronger immune response. ACh is also added because it is the ligand for α7AChR and has been shown to inhibit the expression of TNF-α (Wang et al., 2003). Therefore it is hypothesized that NRG treatments in combination with ACh should lead to a reduction in TNF-α, thus decrease inflammation (Figure 1.6).

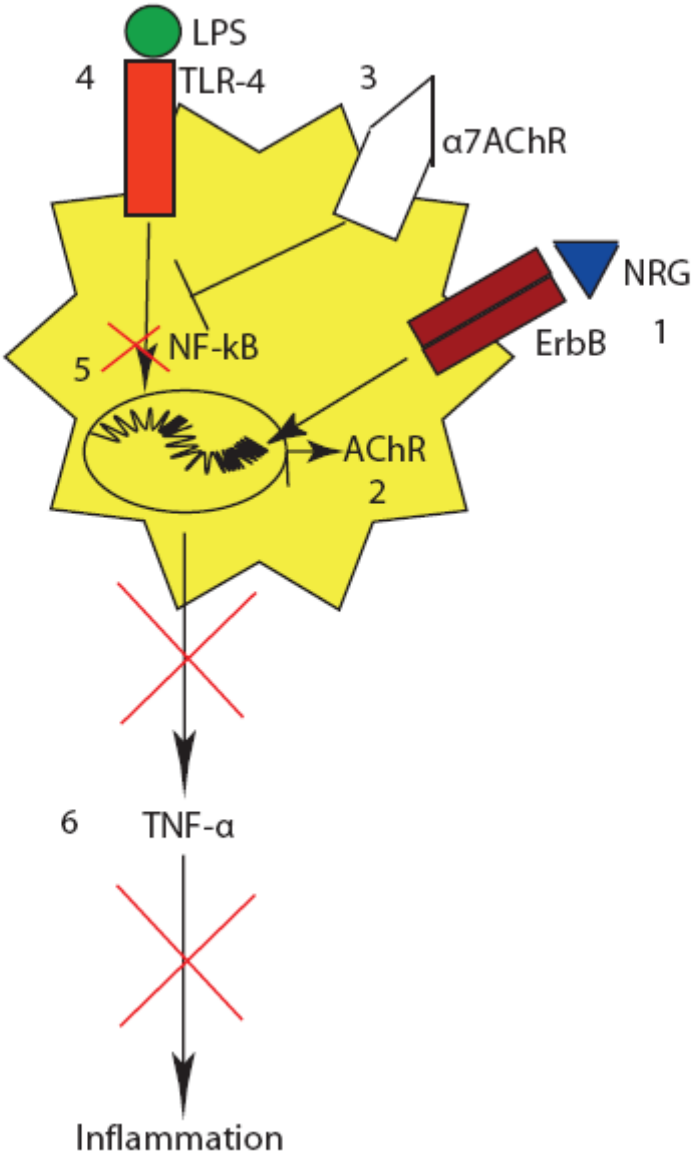


Figure 1.6: Proposed mechanism of NRG action on AChR. Neuregulin signals through the ErbB receptors (1) to increase the transcription of α7AChR (2). The presence of α7AChR decreases the production of TNF-α in response to a foreign invader by inhibiting the NF-κB pathway (3). When microglia become activated through the binding of LPS, present in gram-negative bacteria, to TLR-4 (4) and the subsequent activation of NF-κB, TNF-α that would normally be transcribed is inhibited (5). This elimination of TNF-α affects the inflammatory pathway by hindering the ability of microglia to attract system immune cells and activate surrounding microglia (6).

Chapter 2 Materials and Methods

2.1 Cell culture

2.1.1 Cell lines

EOC-20 cells, a mouse microglial cell line (CRL-2469; ATCC), were cultured in Dulbecco's modified Eagle's medium (Wisent, St. Bruno, QC) with 4mM L-glutamine supplemented with 10% fetal bovine serum (Wisent), 20% LADMAC Conditioned media, 100IU/mL penicillin and 100mg/mL streptomycin (Wisent). Cells were grown at 37°C in a 5% CO_2 in air atmosphere with culture medium renewal every 2 to 3 days. All subsequent passages were performed at a split ratio of 1:3.

The LADMACs, mouse lymphoblast cell line (CRL-2420; ATCC) was cultured for the production of colony stimulating factor-1 (CSF-1). LADMACs were grown in Eagle's Minimum Essential medium (Wisent) supplemented with 10% fetal bovine serum, 100IU/mL penicillin and 100mg/mL streptomycin. Cells were grown at 37°C in a 5% CO_2 in air atmosphere with medium renewal every 2 to 3 days. All subsequent passages were performed at a split ratio of 1:3. Once cells reached confluency, medium was replaced and then not renewed to allow for high concentrations of CSF-1 to build up. At 5 to 7 days, this CSF-1 enriched medium (also known as conditioned medium) was collected and centrifuged at 125 x g for 10 minutes at room temperature. Supernatant was then filter sterilized by passage through a 0.2μM filter and stored at -20°C.

BV-2 is a murine microglial cell line, obtained as a gift from Dr. Michael J. Strong (University of Western Ontario, London, ON). BV-2 cells were cultured in RPMI 1640 (Wisent) supplemented with heat-inactivated fetal bovine serum to a final concentration of 10%, 100IU/mL penicillin and 100mg/mL streptomycin (Wisent). Similar to our other cell lines, cells were grown at 37°C in a 5% CO_2 with medium renewal every 2 to 3 days. When cells were passaged they were split at a 1:5 ratio.

The RAW 264.7 murine macrophage cell line (TIB-71, ATCC) was cultured in a manner similar to BV-2 cells. Passage of these cells was performed at a split ratio of 1:3 to 1:6.

C2C12, a mouse myoblast cell line (CRL-1772; ATCC) was cultured to provide ErbB2 and ErbB3 positive cell extracts (Rimer, 2003). Cells were cultured in Dulbecco's modified Eagle's medium low glucose (Wisent) supplemented with fetal bovine serum to a final concentration of 10%, 100IU/mL penicillin and 100mg/mL streptomycin. Cells were grown at 37°C in a 8% CO_2 in air atmosphere with medium renewal every 2 to 3 days. All subsequent passages were performed at a split ratio of 1:10 when cells reached 80% confluency. Myotubes were cultured in Dulbecco's modified Eagle's medium supplemented with 2% Horse Serum (Wisent), 100IU/mL penicillin and 100mg/mL streptomycin.

Similarly, HeLa S3 cells derived from a human cervical epithelium line (CCl-2.2, ATCC) were cultured to provide extract positive for ErbB4 expression (Sundvall et al., 2008). Culture conditions for HeLa S3 were similar to BV-2 and RAW cells. All subsequent passages were performed at a split ratio of 1:4 to 1:10.

2.1.2 Isolation of microglia from mice

Primary mixed glial cultures were prepared from the cerebral cortices of 1- to 2-day-old C57BL/6 mice. Each culture was derived from a single brain. After the removal of the meninges, the brain was washed in PBS treated with gentamicin, penicillin and streptomycin. The washed cortices were minced and pushed through a 70μm Nylon mesh cell strainer (BD Falcon, Mississauga, ON) followed by filtering through a 40μm Nylon mesh cell strainer (BD Falcon). Cell suspensions were digested in 30mL Hank's balanced salt solution (HBSS) (Invitrogen, Carlsbad, CA) with the addition of DNAse 1 (28U/mL) and collagenase type B (0.2mg/mL) for 1 hour at 37°C at 160rpm. Isolates in HBSS were collected and centrifuged at 800 x g for 10 minutes. Dissociated cells were transferred to a 35mm tissue culture dish (BD Falcon) and cultured in Dulbecco's modified Eagle's medium with 4mM L-glutamine supplemented with 10% fetal bovine serum, 20% LADMAC Conditioned media (CSF-1), 100IU/mL penicillin and 100mg/mL streptomycin. Cells were grown at 37°C in a 5% CO_2 in air atmosphere with medium renewal at 24 hours post-plating and every 2 to 3 days after.

2.1.3 Microglia isolation from mixed glial culture

Two weeks after initiating the culture, supernatant were collected by shaking at 160 rpm following treatment with 10% (v/v) trypsin/EDTA (1:1) (Wisent) for 1 hour at 37°C. Isolates were centrifuged at 800xg for 10 minutes and the resulting cell pellets were

resuspended in growth media and plated on collagen coated plates [0.1% collagen from calf skin (Sigma) and 0.1N acetic acid]. After thirty minutes medium was aspirated to remove any unattached cells, which are mostly astrocytes, and the medium refreshed. Cells were then grown for 24 hours before harvesting.

To assess the purity of primary cell cultures, the assumed pure plate of microglial cells was trypsinized and plated onto glass plates coated with collagen. After a 24 hour incubation period Acetylated Low Density Lipoprotein labeled with 1, 1'-dioctagecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate (BT-902; Biomedical Technologies, Stoughton, MA) was added and incubated for four hours at 37ºC (Voyta et al., 1984**;** Giulian and Baker, 1986) then visualized using a Zeiss Axiovert 200 inverted microscope equipped with Qimaging retiga exi digital camera (Zeiss, Burnaby, BC) to capture the images. Images were then recorded and edited using OpenLab software (PerkinElmer, Waltham, MA).

2.2 Protein extraction

After treatments, cells were briefly washed in ice cold Dulbecco's phosphate buffer saline (D-PBS; Wisent) and harvested with a rubber policeman into 5mL ice cold D-PBS. Cells were spun at 1500rpm for 10 minutes at 4°C, supernatants removed and pellets resuspended in 500µL of protein extraction buffer [25mm Tris pH 7.5, 25mm Glycine, 150mm NaCl, 1x Complete Protease Inhibitor Cocktail (Roche, Mississauga ON), 1% Triton X-100 (Sigma-Aldrich, Oakville, ON) and 5mM EDTA pH 8.0 (Quality Biological Inc, Gaithersburg, MD)]. Isolated cells were incubated on ice for fifteen minutes and then

centrifuged at 4°C for five minutes at 14, 000rpm. Supernatants collected were then either used in immunoprecipitations or electrophoretically separated for immune blotting.

Protein extraction procedures for phosphorylation experiments were performed similar to those outlined in 2.2 with the addition of 1mM sodium orthovanadate (Sigma-Aldrich) to D-PBS and protein extraction buffer.

2.3 Immunoprecipitation

2.3.1 Immunoprecipitation of ErbB receptors

ErbB receptors were immunoprecipitated from samples with equal protein concentrations, determined using the Bradford method (Bradford, 1976), in a total volume of 500µL of lysis buffer. Extracts were incubated with 2µg of rabbit anti-ErBB2, -3 or -4 polyclonal antibody (ErbB2; SC-284, ErBB3; SC-285, ErbB4; SC-283, Santa Cruz Biotechnologies, Santa Cruz, CA) with gentle rocking for 60 minutes at 4°C. The antibody-antigen complex was then incubated with 50µL of Protein G Agarose beads (50% beads/volume; Upstate/Millipore, Billerica, MA) for 60 minutes at 4°C with gentle rocking. The agarose beads were collected by centrifugation at 1000rpm for 15 seconds at 4°C then were washed with 1mL of ice-cold wash buffer (150mm NaCl, 50mm Tris pH 8.0, 1% Triton x100, 1mm Na_3OV_4). Beads were washed five times with centrifugation in between and then resuspended in 50µL of 3x SDS-loading dye; heating for 5 minutes at 95°C. Prepared samples were stored at -20°C until use.

2.3.2 Immunoprecipitation of α7AChR

Cells were first treated as outlined in Table 1 before extraction. For immunoprecipitation of α7AChR receptors, equal amounts of protein (1.0-2.5mg/mL) contained in a total volume of 500µL of lysis buffer were incubated with 1µg of biotin-XX-α-bungarotoxin (Biotium Inc, Burlington, ON) with gentle rocking for 60 minutes at 4°C. The toxin-antigen complexes were then incubated with 50µL of streptavidin beads (Upstate) for 60 minutes at 4°C with gentle rocking. The streptavidin beads were collected by centrifugation at 1000rpm for 15 seconds at 4°C then were washed with 1mL of ice-cold was buffer for a total of five washes with centrifugation in between. Beads were finally resuspended in 50µL of 3x SDS-loading dye and heated for 5 minutes at 95°C. Samples were stored at -20°C until use.

Table 1: Treatments for IP used in ELISA and western blot experiments

Well Label	Treatment, incubation time	Figure
C	No treatment	3.5-3.10
N	1nM NRG, 24 hours	3.5-3.10
L	1mg/mL LPS, 4 hours	3.5-3.10
N+L	1nM NRG, 24 hours 1mg/mL LPS, 4 hours	3.5-3.10
BSA	1mg/mL BSA, 24 hours	3.5
A+N	1nM NRG, 24 hours 500µmol ACh, 23 1/2 hours	3.6, 3.9, 3.10
A+N+L	1nM NRG, 24 hours 500µmol ACh, 23 1/2 hours 1mg/mL LPS, 4 hours	3.6, 3.9, 3.10

Table 1 shows the various treatments used before immunoprecipitation. The IP samples were then subjected to western blotting and ELISA. The corresponding figures to the various treatments are also outlined under the figure heading. NRG treatments were added first to allow for any possible increase in transcription of AChR. ACh was added after NRG treatments to allow for activation of α7AChR. Lastly, was the addition of LPS to initiate an inflammatory response in microglia and macrophages.

2.4 Western blotting

2.4.1 ErbB 2-4 expression

ErbB2-4 expression in microglia and macrophages was confirmed by immunoblot using standard protocols (Jacobson et al., 1998). Protein extracts were electrophoretically separated by an SDS-polyacrylamide gel and subsequently electroblotted onto nitrocellulose membranes. Following blotting, membranes were blocked overnight in 10mM Tris pH 7.5, 0.15M NaCl and 0.1% Tween 20 supplemented with 2.5% BSA/ 2.5% milk. Blots were probed with rabbit anti-ErbB polyclonal antisera (1:1000; Santa Cruz) for 1 hour at room temperature. Specific binding was then visualized using a donkey anti-rabbit horse radish peroxidase (HRP) conjugated antibody (1:1000 dilution, 1 hour at room temperature; Amersham, Arlington Heights, IL) and captured using chemiluminescence detection ECL (Pierce, Rockford, IL) on autoradiography film (GE, Mississauga, ON). Anti-mouse beta-tubulin (gift from Dr. Mungo Marsden, University of Waterloo, ON) was used (1:5000 dilution, 1 hour at room temperature) to ensure equal loading and presence of protein in crude cell extracts. Beta-tubulin immunoblots were ran in parallel with anti-ErbB antibodies and were visualized using rabbit anti-mouse HRP conjugated antibody (1:2000 dilution, 1 hour room temperature; Amersham).

2.4.2 Activation of ErbB receptors in the presence of neuregulin

ErbB IP samples were electrophoretically separated on 8% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane as described above (2.4.1). For

phosphotyrosine detection, blots were probed with the 4G10 monoclonal anti-phosphotyrosine antibody (1:1000 dilution, 1 hour room temperature; Upstate Biotechnology, Lake Placid, NY) in buffer containing 10mM Tris pH 7.5, 0.15M NaCl, 0.1% Tween 20 and0.05% IGPAL supplemented with 5% BSA**.** Specific binding was then visualized using a rabbit anti-mouse horse radish peroxidase (HRP) conjugated antibody (1:2000 dilution, 1 hour room temperature; Amersham).

2.4.3 Alpha seven acetylcholine receptor expression

Alpha 7 AChR expression was confirmed by immunoblot using standard protocols outlined previously (2.4.1). Blots were probed using rabbit anti-α7AChR polyclonal antibody (1:500, 1 hour at RT; sc-1447, Santa Cruz) and visualized using a donkey anti-rabbit horse radish peroxidase (HRP) conjugated antibody (1:1000 dilution, 1 hour at RT; Amersham).

2.5 Enzyme Linked Immunosorbent Assay (ELISA)

To determine if microglia are activated in response to neuregulin treatment the concentration of TNF-α in cell culture media was determined by ELISA.
At varying time points, medium was harvested from treated plates (Table 1). TNF-α concentration was measured using Mouse TNF-α ELISA Ready-SET-Go kit (88-7324; eBioscience, San Diego, CA) and performed according to procedure accompanying ELISA kit. Briefly, 96 well Corning Constar 9018 plates were coated with 100μL/well of capture

antibody in coating buffer, sealed with parafilm and incubated overnight at 4°C. Plates were then aspirated and washed five times with 250µL/well of wash buffer (1x PBS and 0.05% Tween-20). Wells were then blocked with 200µL/well of 1X assay diluent. After incubation for 1 hour at room temperature wells were again washed five times. Next, 100µL/well of TNF-α of known concentration at varying dilutions in 1x assay diluent was added to wells to create a standard curve (appendix A). Experimental samples were also added at 100µL/well. The plate was then covered with parafilm and incubated overnight at 4°C. After aspirating and washing five times 100µL/well of detection antibody diluted in 1X assay diluent was added, covered and incubated at room temperature for 1 hour. Plates were then aspirated and washed five times. Avidin-HRP diluted in 1X assay diluent was added at 100µL/well, covered and incubated at room temperature for 30 minutes. Wells were then soaked in wash buffer for 2 minutes and aspirated for a total of seven washes. Lastly, 100µL/well of substrate solution was added and the plated was incubated at room temperature for 15 minutes before the addition of 50µL/well of stop solution. Plates were then read at 450nm using a microplate reader.

Chapter 3 Results

3.1 Isolation and identification of microglia from mixed cell populations harvested from brain tissue

There are inconsistencies in the literature regarding which ErbB receptors are expressed by microglia (Table 2). This contradictory information appears to depend on the cells cultured; with primary and immortalized cells producing divergent results (Dimayuga et al., 2002; Gerecke et al., 2001; Chaudhury et al., 2003; Cannella et al., 1999). Due to these discrepancies, and the importance of the ErbB receptors in neuregulin signaling, my initial investigation analyzed ErbB receptor expression in both primary cells and immortalized cell lines.

Table 2: Reports of ErbB receptors in microglia

Author, year	ErbB receptor present	Cell type
Xu and Ford, 2005	ErbB4	Primary
Dimayuga et al., 2003	ErbB2-4	N9 (immortalized cell line)
Chaudhury et al., 2003	ErbB4 and ErbB2	Primary isolated from neuritic plaques in Alzheimer's disease
Gerecke et al., 2001	ErbB3 and ErbB4	Primary
Erlich et al., 2002	No early ErbB4 until after injury	Primary, conclude that expression is due to phagocytosis
Canella et al., 1999	ErbB3, ErbB2 inconclusive ErbB4 in MS	Normal primary and MS

Table 2 illustrates the current literature with regards to ErbB expression in microglia. Both immortalized cells lines and primary cells are represented. ErbB4 expression is solely seen in damaged and diseased tissues. Immortalized cell line, N9, shows expression of ErbB2-4 receptors. Primary cells show the largest variation with ErbB expression.

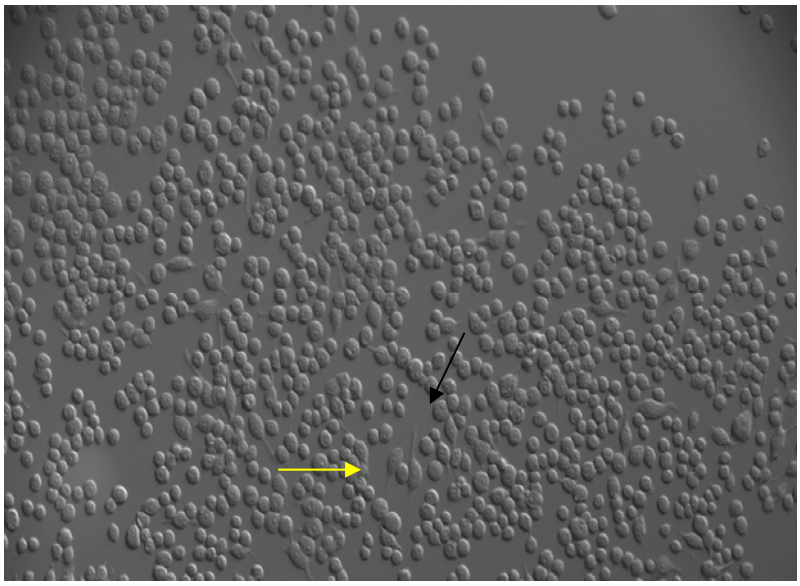
Adult and neonate primary microglial cultures were isolated from the brains of 8 week and P1 to P3 day old mice respectively. Cells isolated from mouse brains were grown for two weeks as mixed glial populations prior to purification. Adult microglial cells, when isolated, did not grow very well in culture compared to those isolated from neonates

(Figure 3.1) and as such were not used extensively. Nonetheless, adult primary cells, when extracted and immunoblotted for ErbB receptor expression were shown to not express any of the ErbB family of receptors (data not shown).

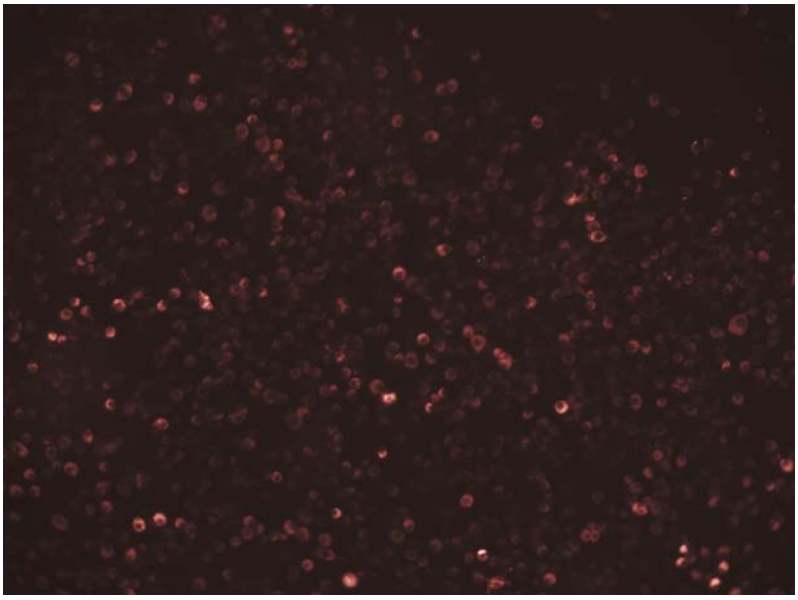
Purity of cell cultures was determined by labeling putative microglia with Dil labeled Ac-LDL, a microglia specific fluorescent marker for phagocytosis (Figure 3.1, B and C). Images were taken at 10x (Figure 3.1, A and B**)** and 40x magnification (Figure 3.1, C) to analyze cell morphology and purity obtained though percentage of fluorescence uptake**.** Analysis of fluorescent uptake quantified from DIC and fluorescent fields of 100 cells over 3 experiments indicated that the primary microglia cultures were 98% pure +/- 1%. At this level of purity there is very little potential for artifactual errors to be introduced by contaminating cells; in this case 1-2% is astroctyes. In my purified microglia, I saw the presence of ramified microglia. Existing in 5% of the total purified microglia population, ramified microglia are characterized by processes protruding from their soma. Contrasting this are amoeboid cells which appear circular in appearance. In addition to differences in morphology, ramified cells phagocytose more fluorescence and appear redder than amoeboid cells.

Figure 3.1 Establishing the purity of neonate microglia obtained from mixed glial cell populations. Differential interference contrast microscopy at 10x magnification of pure neonate microglia was used to show the morphology of microglia (A). Microglial cells appear uniform in size, shape and morphology. Most putative microglia appear to be amoeboid (indicated by black arrow) while approximately 5% of cells appear to be ramified, identified by processes protruding from the soma (yellow arrow)**.** A rhodamine filter set was used to image Dil phagocytosis by microglia. Images have been edited via Adobe Photoshop (Adobe Systems Canada, Toronto, ON) to show red corresponding to uptake of fluorescence (B and C).The image shown in A has a corresponding fluorescence image (B)**.** Red puncta indicated phagocytosis of Dil-Ac-LDL identifying cells as microglia. Absence of cells without red puncta confirms successful isolation of microglia cells. DIC and fluorescence images were juxtaposed at 40x magnification (C) to confirm purity. Primary microglia (C) exists in two forms: amoeboid (indicated by yellow arrow) and ramified (indicated by green arrow**).** Ramified microglia uptake more fluorescence and appear redder than amoeboid microglia.

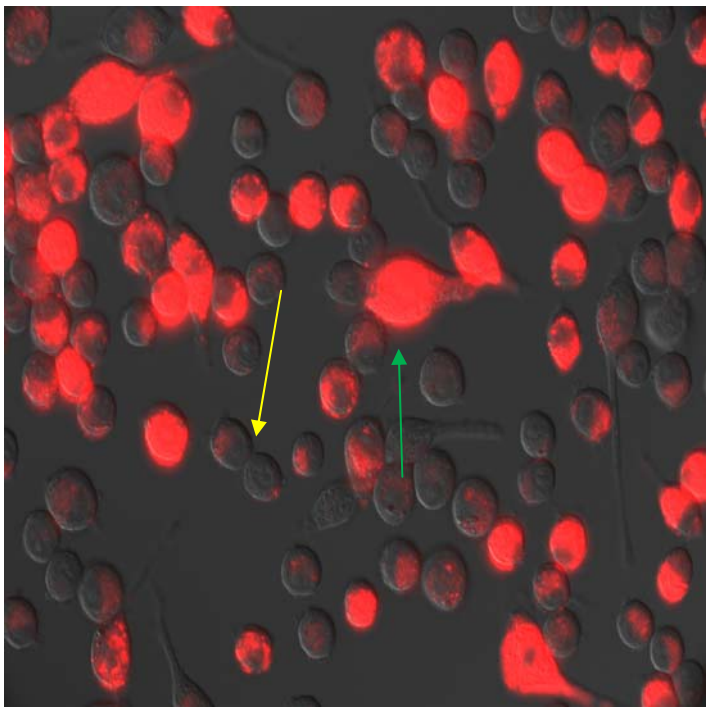
A



B



C



3.2 ErbB expression in microglial and macrophage cell lines

The ErbB receptors are a family of tyrosine kinase receptors that become phosphorylated upon ligand binding (Buonanno and Fischbach, 2001). To determine if the ErbBs are expressed and verify NRG's ability to stimulate the tyrosine phosphorylation of these receptors, I cultured the murine cell lines, EOC-20, BV-2 and RAW 264.7, as well as microglia isolated from postnatal mice as previously described. The crude cell extracts where then analyzed by immunoblot for ErbB2-4 receptor expression (Figure 3.2 and Figure 3.3).

In figure 3.2 there is an absence of banding at the molecular mass expected for the ErbB receptors. Whole cell extracts of all three immortalized cell lines appear to not express any of the ErbB receptors (Figure 3.2) or do so below the threshold of detection of this assay. Mouse anti-beta tubulin antibody was also used to ensure that absence of banding was due to an absence or low levels of expression and not due to inadequate levels of protein loaded into each well.

To determine if ErbB expression is below the threshold of detection in my crude samples I immunoprecipitated with the various ErbB antisera to purify the ErbB receptors from my extracts. In immunoblots of immunoprecipitated samples, ErbB4 is clearly seen in EOC-20, BV-2 and RAW 264.7 cells (Figure 3.4). This is a different result from that obtained in figure 3.2 where there was an absence in banding at 185kDa representing ErbB4 receptors in EOC-20, BV-2 and RAW cells. The expression of ErbB4 in the previously mentioned cell lines in figure 3.4 is due to the increased concentration of

receptors via immunoprecipitation to allow for low levels of expression that may not appear in crude cell extracts.

In figure 3.3 there is expression of all three ErbB receptors in primary microglial cells. Both positive controls (C2 and HeLa) and primary cells show banding at 185kDa indicative of ErbB2-4 expression.

Having determined that ErbB4 is in fact expressed I endeavored to determine if these receptors are activated by NRG. To do this extracts isolated from NRG treated and control cultures were immunoprecipitated with anti-ErbB antisera and then immunoblotted with the anti-phosphotyrosine antibody 4G10 (Figure 3.4). NRG addition should show an increase in ErbB phosphorylation over cells left untreated, this is indeed the case where I saw ErbB4 phosphotyrosylation (Figure 3.4, bottom panel).

Figure 3.2 Absence of ErbB2-4 receptor expression in whole cell lysate microglia and macrophage cell lines. Immunoblots of whole cell lysate from microglial cell lines (EOC-20 and BV-2) and macrophage (RAW 264.7) were probed with antibodies specific for each ErbB receptor. Arrows on the right of each panel indicate the position of each expected immunoreactive species and its mass (185 kDa). The anti-ErbB2 antibody used for the immunoblot (top panel) was a rabbit polyclonal antibody specific for the ErbB2 carboxy terminus (sc-284; Santa Cruz Biotechnologies, Santa Cruz CA). Subsequently, the anti-ErbB3 antibody (sc-285) and anti-ErbB4 antibody (sc-283) were also used (middle panel and bottom panel respectively). The upper bands at approximately 185kDa represent the respective receptor bands (indicated by arrow). Positive control cell lines were C2C12 for ErbB2 and 3 and HeLa S3 (H) for ErbB4. Secondary controls (shown at right) were probed with secondary antibody only. Mouse anti-beta-tubulin antibody was used to ensure equal loading of cell extracts indicated by the band at the bottom of each ErbB panel at 50kDa. EOC-20, BV-2 and RAW 264.7 cells are devoid of banding at 185kDa indicating a possible absence or low levels of ErbB2-ErbB4 receptor expression.

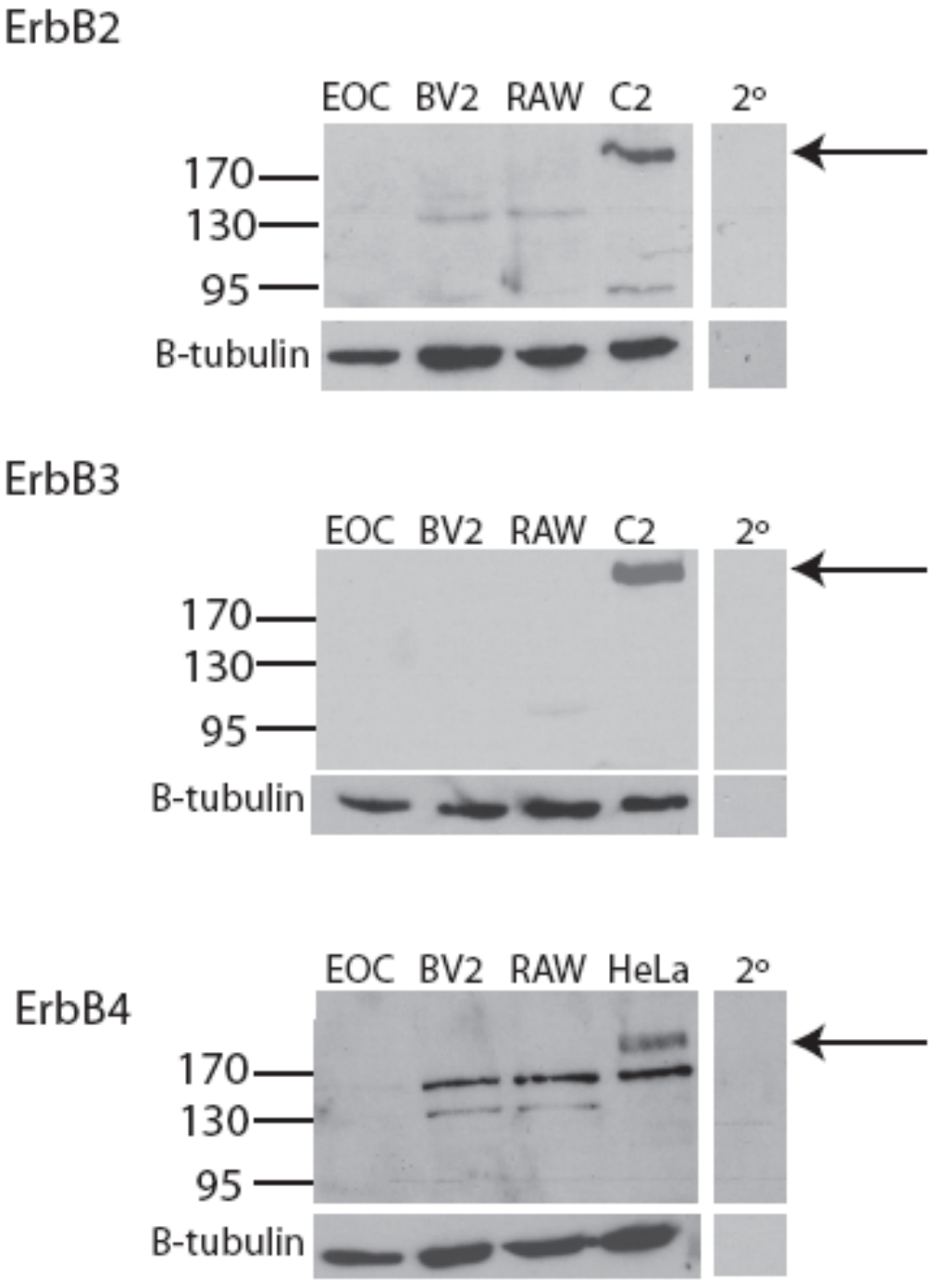

ErbB2
EOC
BV2
RAW
C2
2°
170
130
95
B-tubulin
ErbB3
EOC
BV2
RAW
C2
2°
170
130
95
B-tubulin
ErbB4
EOC
BV2
RAW
HeLa
2°
170
130
95
B-tubulin

Figure 3.3 ErbB2-4 are expressed in primary microglia harvest from mice.

Immunoblots of whole cell lysate from microglial cells harvested from brains of P1 to P3 day old mice (P) were probed with antibodies specific for each ErbB receptor. Each lane of extracts were obtained from one 10cm dish of confluent cells. Arrows on the right of each panel indicate the position of each expected immunoreactive species and its mass (185 kDa). The anti-ErbB2 antibody used for the immunoblot (top panel) was a rabbit polyclonal antibody specific for the ErbB2 carboxy terminus (sc-284; Santa Cruz Biotechnologies, Santa Cruz CA). Subsequently, the anti-ErbB3 antibody (sc-285) and anti-ErbB4 antibody (sc-283) were also used (middle panel and bottom panel respectively). The upper bands at approximately 185kDa represent the respective receptor bands**.** Positive control cell lines were C2C12 for ErbB2 and 3 and HeLa S3 (H) for ErbB4. Secondary controls (shown at right) were probed only with the secondary antibody, anti-rabbit HRP. ErbB2-4 expression is seen in primary microglia as indicated by the presence of banding at 185kDa (indicated by arrow).

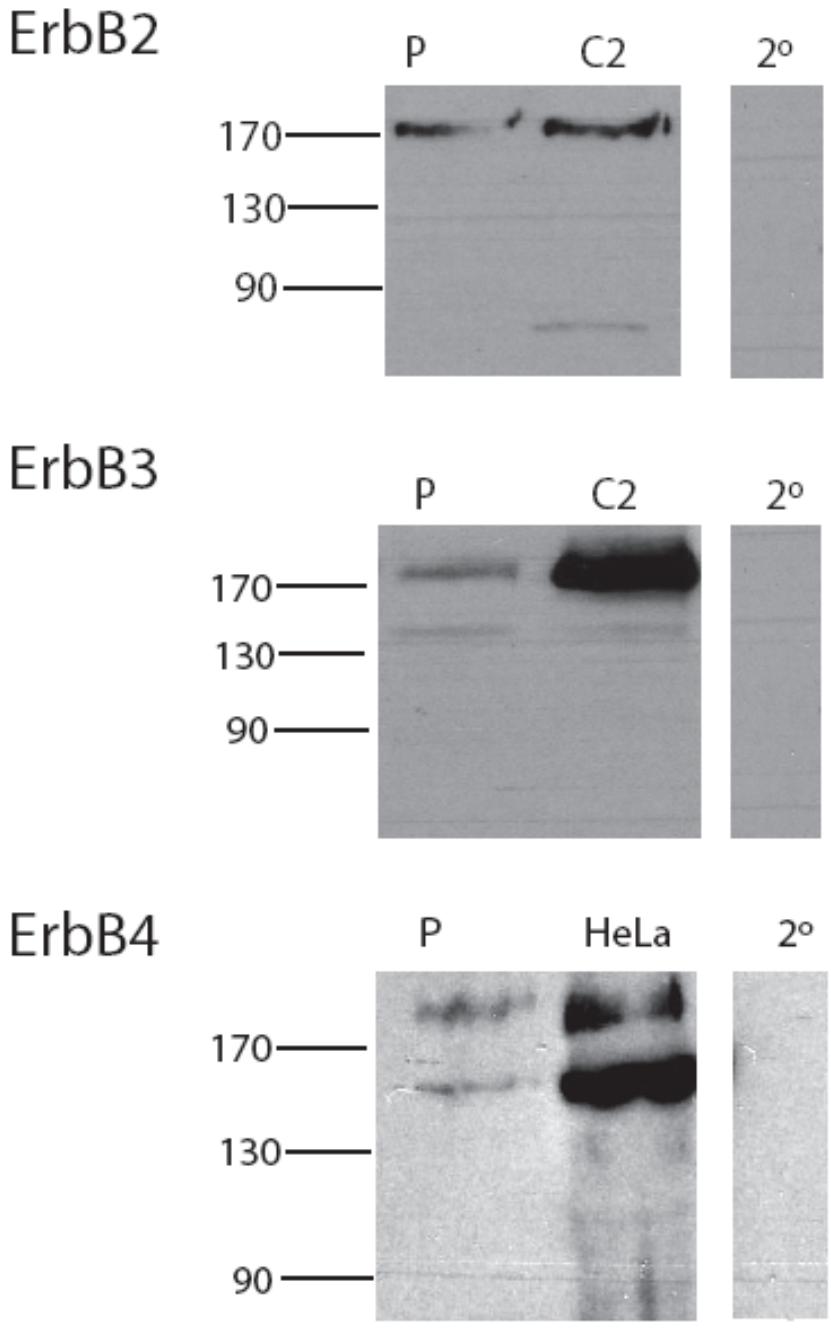
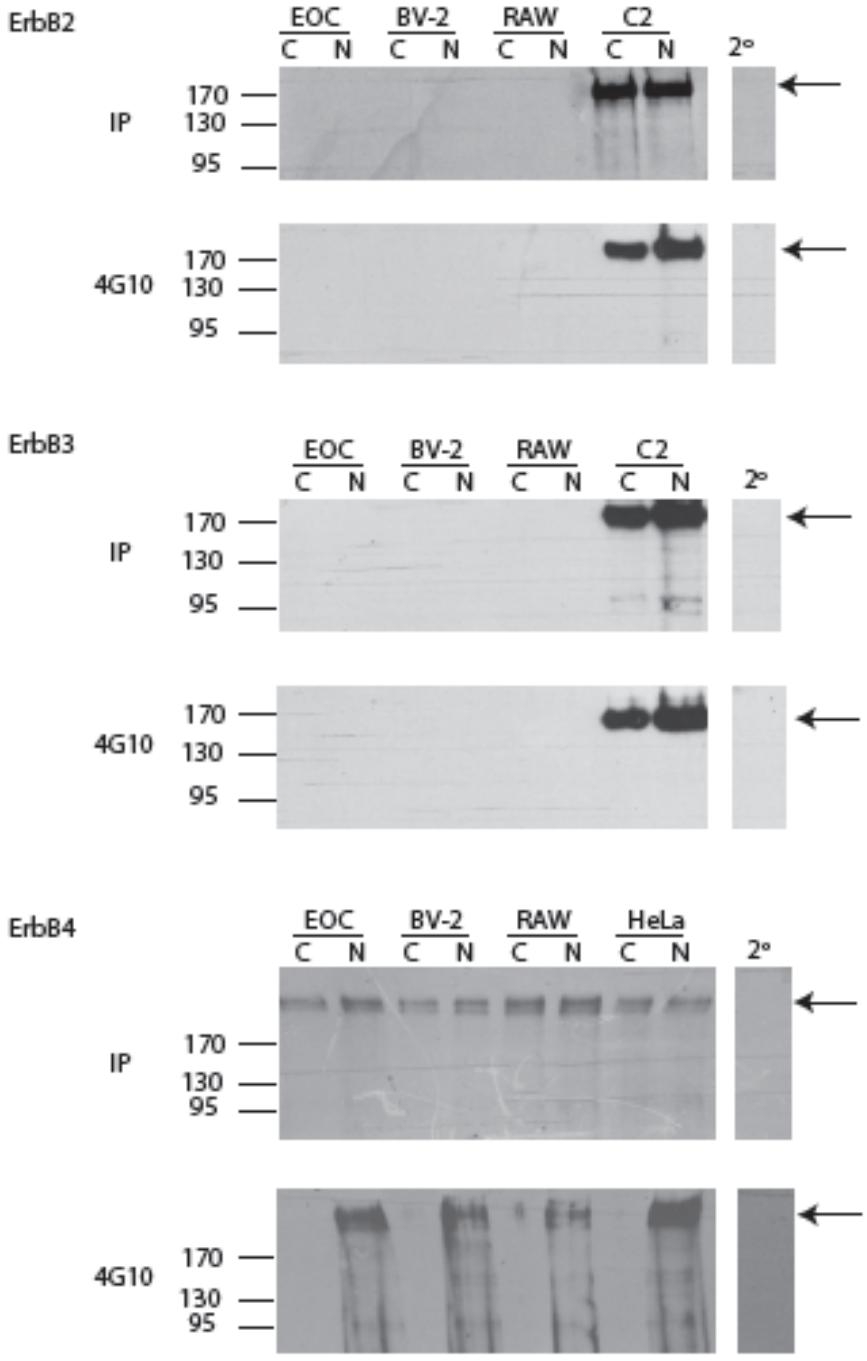

ErbB2
P
C2
2°
170
130
90
ErbB3
P
C2
2°
170
130
90
ErbB4
P
HeLa
2°
170
130
90

Figure 3.4 Expression and phosphorylation of ErbB4 receptors in microglia and macrophage cell lines. NRG treated (N) and untreated (C) samples of microglia (EOC and BV-2) and macrophage (RAW) cell lines were immunoprecipitated to increase the concentration of ErbB receptors. Blots were first probed with antibodies specific for phosphotyrosylation (4G10, bottom panel). The anti-phosphotyrosine antibody used for the immunoblot was a mouse monoclonal antibody (4G10; upstate signaling). Blots were stripped and then reprobed with anti-ErbB2-4 antibodies (IP, top panel). Positive control cell lines were C2C12 (C2) for ErbB2 and 3, and HeLa S3 (H) for ErbB4. Negative controls were shown right. Beads alone were also immunoprecipitated (data not shown). Immunoprecipitated samples (IP) are shown on top with the phosphorylated immunoblot (4G10) on the bottom for each receptor. EOC-20, BV-2 and RAW 264.7 cell lines do not possess banding at 185kDa indicative of ErbB2 and ErbB3 expression and phosphorylation (ErbB2 and ErbB3). ErbB4 blots show equal size bands in all immunoprecipitated samples (top of ErbB4) indicating that ErbB4 is expressed in EOC, BV-2 and RAW cells. 4G10 blot of ErbB4 IP samples show a stronger banding with NRG treated samples than untreated indicating that NRG is phosphorylating ErbB4 receptors. Secondary controls (indicated by 2˚) followed same experimental procedures as the other wells but were not probed with anti-ErbB antibodies.


ErbB2
EOC
BV-2
RAW
C2
C N C N C N C N
2°
IP
170
130
95
4G10
170
130
95
ErbB3
EOC
BV-2
RAW
C2
C N C N C N C N
2°
IP
170
130
95
4G10
170
130
95
ErbB4
EOC
BV-2
RAW
HeLa
C N C N C N C N
2°
IP
170
130
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4G10
170
130
95

3.3 Neuregulin increases α7AChR expression in microglia

In skeletal muscle neuregulin regulates AChR transcription via the ErbB receptors (Buonanno and Fischbach, 2001). To determine if NRG has the ability to increase α7AChR transcription in microglia and macrophage cells, as it does in muscle, immunoblot analysis was performed on extracts isolated from primary cells and cell lines. Cultures were treated with NRG, LPS and NRG and LPS (as outlined in table 1) and α7AChR were purified by precipitation with a combination of α-BTX biotin and streptavidin agarose beads. Immunoblot analyses were then performed with α7AChR antisera to determine if expression changed with treatment (Figures 3.5 and 3.6)**.**

In RAW cells I saw no statistically significant changes in α7AChR expression with my treatments. RAW cells treated with NRG increased α7AChR concentration 1.11± 0.49 fold over untreated cells. Treatment with LPS increased 1.16±0.73 fold and NRG combined with LPS increased 1.25±0.19 fold over untreated cells. Combined, these results demonstrate that RAW cells showed no difference in α7AChR expression, even in the presence of NRG.

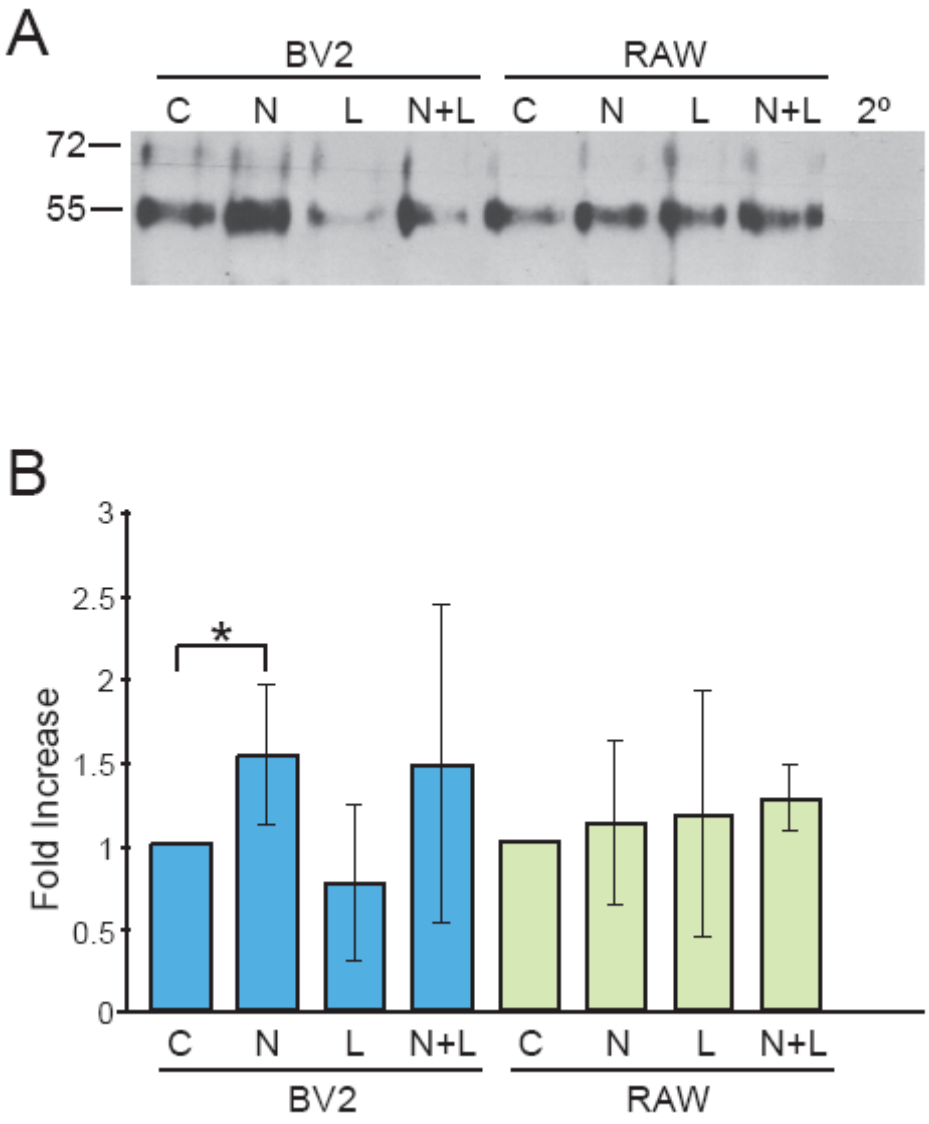
Contrasting this were the results obtained in BV-2 cells. BV-2 cells when treated with NRG overnight showed an approximate 1.53±0.42 fold increase in α7AChR expression ($n=3$, $p<0.05$, t-test; Figure 3.5 B). BV-2 cells appeared to show a decrease in α7AChR expression when treated with LPS (Figure 3.5A) but when statistical analysis was applied, these results were inconclusive (0.76 ± 0.47 compared to untreated cells). The difference in neuregulin and LPS treated BV-2 cells were also not statistically

significant due to a high variability in these experiments. These results taken together show that NRG does increase α7AChR expression in BV-2 cells.

The expression of α7AChR was also analyzed via immunoblot in primary cells harvested from P3 day old mice (Figure 3.6**)**. Similar to the results obtained from BV-2 cells, NRG addition increased α7AChR expression (Figure 3.6). Untreated primary cells had undetectable levels of α7AChR expression.

As can be seen, the treatments were similar to those in my earlier microglial and macrophage cells line experiments but with two additional treatments; acetylcholine was added in addition to NRG and added to NRG+ LPS (as outlined in table 1). Acetylcholine addition was pertinent to the expression of α7AChR in LPS and NRG treated samples. NRG and LPS treated microglia showed expression of α7AChRs when ACh was added to the treatment (ACh +N+L, Figure 3.6) oppose to zero expression in its absence (N+L, Figure 3.6). Interestingly, ACh addition increased α7AChR expression in primary cells treated with NRG and LPS. NRG also appears essential for expression of α7AChR in primary microglial cells where α7AChR expression in normally absent or expressed at low levels in untreated cells.

Figure 3.5 NRG increases α7AChR expression in microglia and macrophages. Microglia and macrophage cell cultures were treated overnight with NRG (N) or left untreated (C). LPS (L) was added twenty four hours later for a period of four hours before extraction (table 1). After treatment, cells were toxin-precipitated with biotin conjugated α-BTX and streptavidin agarose beads. Immunoblots were probed with a rabbit anti-α7AChR polyclonal antibody (1:500 dilutions, Santa Cruz) and visualized using a donkey anti-rabbit HRP conjugated antibody (1:1000 dilution, Amersham). The lower bands at approximately 55kDa represent α7AChR. Densitometry analysis of immunoblot (A) shows a 1.53 ± 0.42 fold increase in α7AChRs over control in NRG treated BV-2 cells ($n=3$, $p < 0.05$, t-test). BV-2 cells appeared to show a decrease in α7AChR expression when treated with LPS (A) but when statistical analysis was applied, these results were inconclusive (0.76 ± 0.47 compared to untreated cells). Neuregulin and LPS treated BV-2 cells also varied greatly in their results (1.47±0.95 fold increase). These results taken together show that NRG does increase α7AChR expression in BV-2 cells. RAW cells treated with NRG showed a 1.11± 0.49 fold increase over untreated cells. Treatment with LPS yielded a 1.16±0.73 fold increase and NRG combined with LPS showed a 1.25±0.19 increase over untreated cells. Combined, these results demonstrate that RAW cells showed no difference in α7AChR expression, even in the presence of NRG. Error bars represent standard deviation.


A
BV2
RAW
C
N
L
N+L
C
N
L
N+L
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72
55
B
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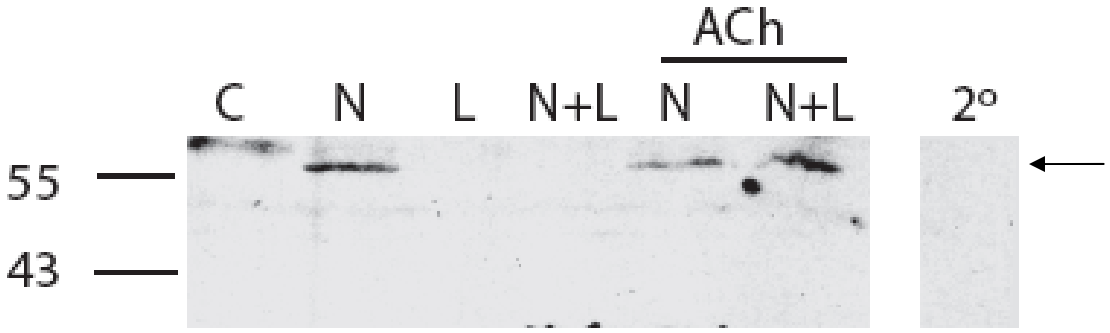
3
2.5
2
1.5
1
0.5
0
*
C
N
L
N+L
C
N
L
N+L
BV2
RAW

Figure 3.6 NRG increases α7AChR expression in primary microglia. Primary cells harvested from brains of P3 day old mice were treated for twenty four hours with NRG. Thirty minutes after NRG addition (indicated by N), acetylcholine was added in the last two wells (indicated by ACh label). The following day LPS was added (indicated by L) for four hours before protein extraction. After extraction, cells were toxin-precipitated with biotin conjugated α-BTX and streptavidin agarose beads. Immunoblots were probed with a rabbit anti-α7AChR polyclonal antibody. The upper bands at approximately 55kDa (indicated by arrow) represent the α7AChR. Untreated cells (labeled C) showed an absence of banding along with N and L treated samples. NRG treated cells showed presence of α7AChR. Both ACh treated samples also showed α7AChR expression. Secondary control (shown right) contains the same concentration of protein loaded into the well but was not probed with anti-α7AChR polyclonal antibody.


ACh
C
N
L
N+L
N
N+L
2°
55
43

3.4 The concentration of TNF-α remains unaffected by NRG addition

TNF-α is a pro-inflammatory cytokine released by microglia in response to an assault to the CNS (Wang et al., 2003). De Simone and colleagues (2005) have shown that increases in α7AChR expression can lead to a decrease in TNF-α. In section 3.3 I showed that NRG had an effect on α7AChR expression in BV-2 and primary cells. Since α7AChR expression has been inversely related to the expression of TNF-α (Wang et al., 2003; De Simone et al., 2005), it is possible that NRG in conjunction with ACh might reduce TNF-α expression in my microglia cultures.

Media extracted from the cultures treated with NRG (24 hours), LPS (4 hours) and NRG + LPS (24 hours and 4 hours respectively) were analyzed for TNF-α expression. Cell culture medium was taken at 10 minutes, 30 minutes and 240 minutes post treatment. Culture medium was refreshed prior to these experiments to evaluate basal level expression and to accurately measure effects of the treatments (time = 0). Finally to ensure that NRG treatments alone were not inflammatory, I used BSA as a control treatment (treated for 24 hours) to identify unintentional activation of microglia.

Medium was analyzed for TNF-α using an ELISA (Figures 3.7-3.10). Concentrations of TNF-α were calculated using a standard concentration curve. This curve was constructed by performing two fold serial dilutions of a known concentration of TNF-α (1000pg/mL) and plotted on a log-log scale (Figures A.1 and A.2). Slope of the standard curve was then used to calculate TNF-α concentration in Figures 3.7 to 3.10.

Acetylcholine is a ligand for α7AChR and its effects on TNF-α were also measured with respect to NRG. It is possible that NRG is able to increase α7AChR

expression but is unable to have an effect on the anti-inflammatory response unless ACh is present. To account for this, the effects of ACh in addition to the previous treatments were investigated using an ELISA (Figures 3.9 and 3.10). It has been previously established that NRG increases α7AChR (Figures 3.5 and 3.6), therefore the addition of ACh to NRG treated samples might reduce the concentration of TNF-α when treated with LPS. This is also assuming that my microglial cultures follow similar patterns to the microglial cells used by De Simone and colleagues (De Simone et al., 2005). This is not the case as seen below and in figures 3.9 and 3.10.

In figure 3.7 I have shown that in RAW cells, the addition of NRG twenty four hours prior to LPS treatment does not have an effect on the concentration of TNF-α (610 ±15.98 pg/mL) compared to LPS alone (640 ±4.04 pg/mL) (n=2). However, in LPS treated cells the concentration of TNF-α increased compared to untreated cells (29.47±1.58 pg/mL, n=2). Therefore in RAW cells, NRG does not have an affect on α7AChR (Figure 3.5) or on TNF-α expression (Figure 3.7).

Similar to RAW cells, LPS addition in BV-2 cells resulted in an increase in the concentration of TNF-α (361.34±179.17 pg/mL) compared to untreated cells (13.98±1.82 pg/mL) at 240 minutes (n=2; Figure 3.9). The concentration of TNF-α also increased with NRG and LPS treatments over untreated cells however the addition of NRG in LPS samples did not affect TNF-α concentration (371.35±213.6 pg/mL, n=2; Figure 3.9) Surprisingly, the addition of ACh to cells treated with NRG did not affect the concentration levels of TNF-α (308.56±43.79 pg/mL) when stimulated with LPS compared to cells that did not receive ACh (371.35±213.6 pg/mL) at 240 minutes (n=2).

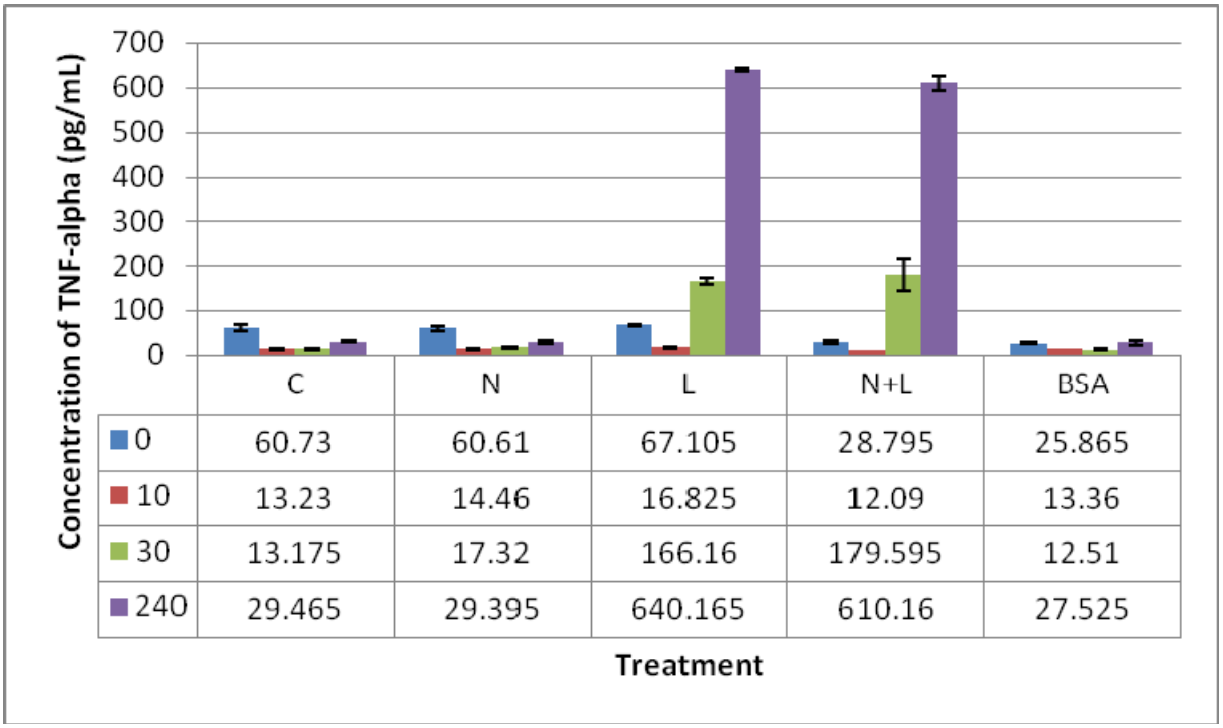
Therefore in BV-2 cells, NRG regardless of ACh addition does not appear to have an effect on TNF-α expression.

Similar findings were seen in primary microglia (Figure 3.10) as seen in BV-2 cells (Figure 3.9). LPS addition resulted in an increase in the concentration of TNF-α (586.04±17.72 pg/mL) compared to untreated cells (7.03±1.33 pg/mL) at 240 minutes (n=2).The concentration of TNF-α remained unchanged with the combination of NRG and LPS versus treatments of LPS alone (553.96±23.08 pg/mL, n=2). The addition of ACh to cells treated with NRG did not affect the concentration levels of TNF-α (547.66±9.66 pg/mL) when stimulated with LPS compared to cells that did not receive ACh (553.96±23.08 pg/mL) at 240 minutes (n=2).

In summary, ACh addition does not reduce the amount of TNF-α in NRG treated primary or BV-2 cells (Figures 3.9 and 3.10). Despite seeing increases in α7AChR when treated with NRG, this increase does not correspond to a decrease in TNF-α. Additionally, NRG does not have an effect on TNF-α concentration in RAW cells (Figure 3.7) as expected since NRG also does not affect α7AChR expression in macrophages (Figure 3.5).

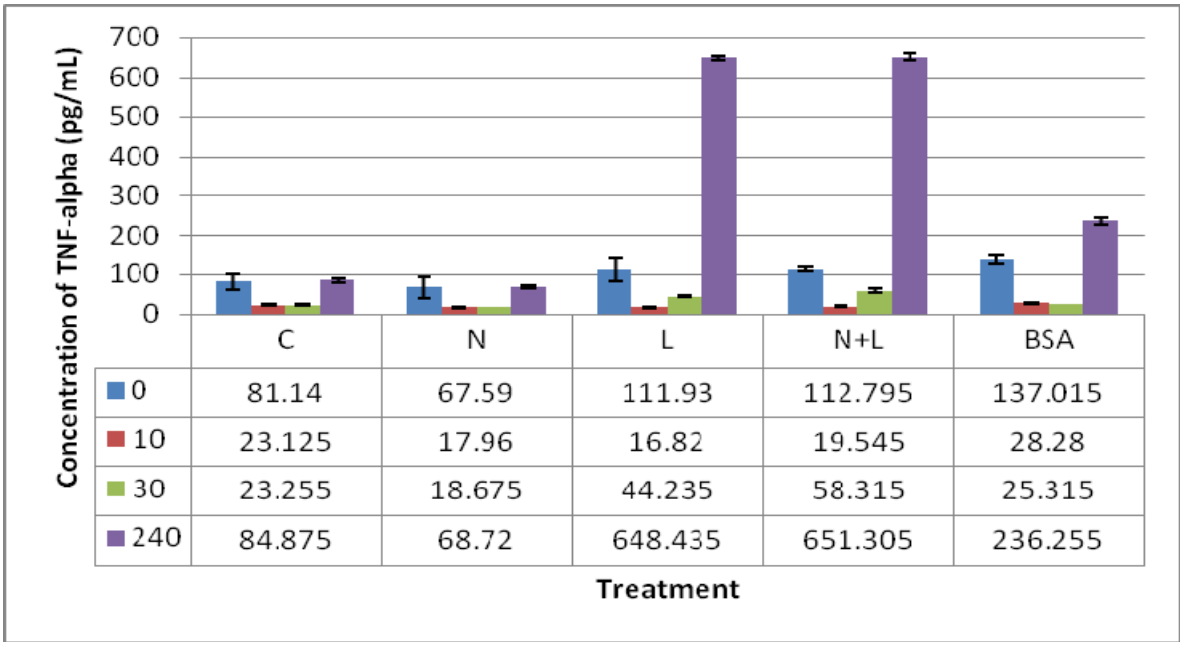
Figure 3.7 NRG does not affect TNF-α expression in macrophages. RAW 264.7 cells were the representative macrophage cell line used to show the various treatment effects on TNF-α. A standard curve of TNF-α was constructed to determine the concentration levels of TNF-α in RAW cells (Figure A.1). Samples with basal levels of TNF-α were taken before the addition of various treatments to determine resting levels of the cytokine (indicated by 0). Untreated cells (indicated by C) and BSA (BSA) are used as controls for this experiment. Cells were treated with NRG (N) for 24 hours, LPS (L) for 4 hours and BSA for 24 hours.

There was a minimal level of TNF-α present in cells before treatment. The addition of NRG 24 hours prior to LPS treatment did not have an effect on the concentration TNF-α (610 ±15.98 pg/mL) compared to LPS alone (640 ±4.04 pg/mL) (n=2). LPS treated cells did increase the concentration of TNF-α compared to untreated cells (29.47±1.58 pg/mL, n=2). Error bars represent standard deviation.



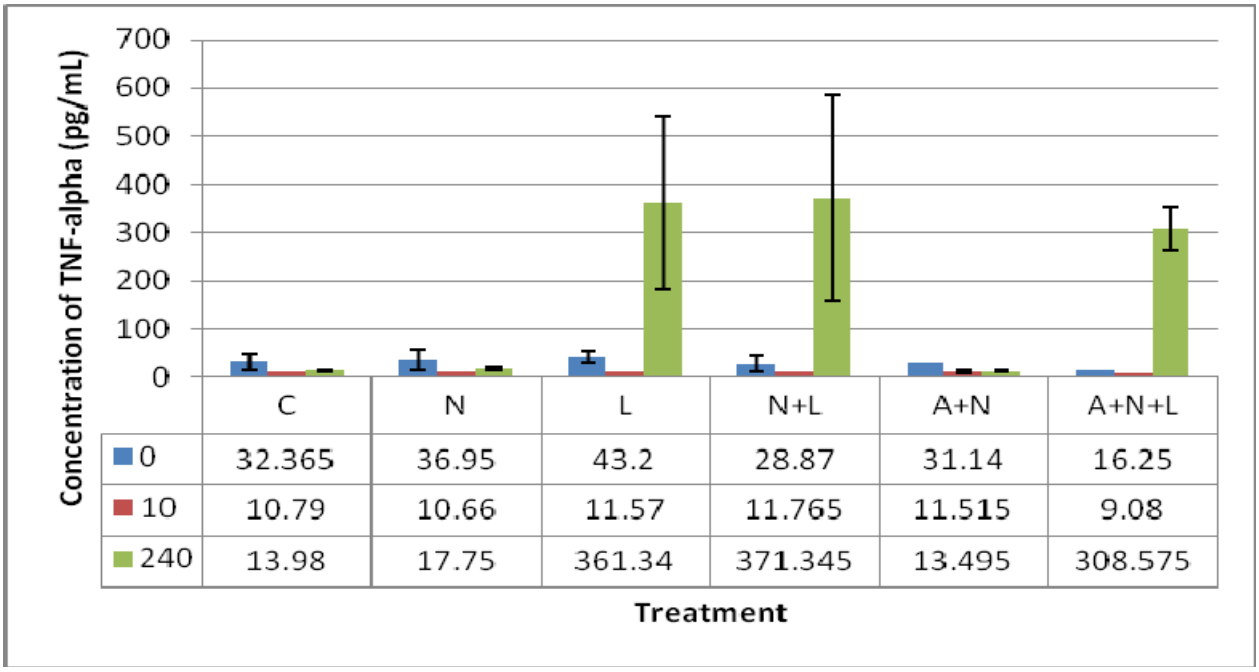
	C	N	L	N+L	BSA
0	60.73	60.61	67.105	28.795	25.865
10	13.23	14.46	16.825	12.09	13.36
30	13.175	17.32	166.16	179.595	12.51
240	29.465	29.395	640.165	610.16	27.525

Figure 3.8 NRG has no effect on TNF-α expression in microglia. BV-2 cells were the representative microglial cell line used to show the various treatment effects on TNF-α. All values were calculated using the slope of a standard curve of TNF-α at varying dilutions (Figure A.1). Basal levels of TNF-α were taken before the addition of various treatments to eliminate out resting levels of the cytokine (indicated by 0). Untreated cells (indicated by C) and BSA (BSA) are used as controls for this experiment. Cells were treated with NRG (N) for 24 hours, LPS (L) for 4 hours and BSA for 24 hours. There is a notable base level of TNF-α which is present before treatment. The addition of LPS caused an increase in the concentration of TNF-α (648.44± 5.96 pg/mL) over untreated cells (84.88±4.05 pg/mL) at 240 minutes (n=2). The concentration of TNF-α remained unchanged regardless of NRG addition to LPS treated cells (651.39±7.88 pg/mL, n=2). Error bars represent standard deviation.



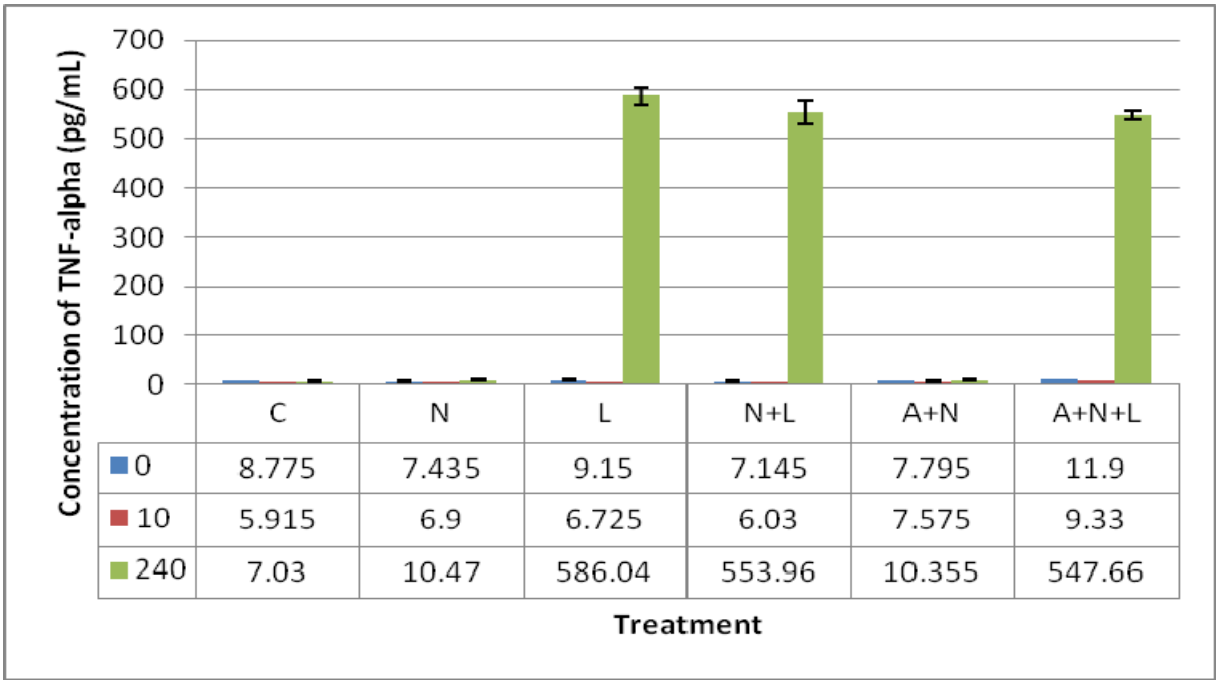
	C	N	L	N+L	BSA
0	81.14	67.59	111.93	112.795	137.015
10	23.125	17.96	16.82	19.545	28.28
30	23.255	18.675	44.235	58.315	25.315
240	84.875	68.72	648.435	651.305	236.255

Figure 3.9 TNF-α expression in BV-2 cells are unaffected by NRG addition even in the presence of acetylcholine. All values were calculated using the slope of a standard curve of TNF-α at varying dilutions (Figure A.2). Basal levels of TNF-α were taken before the addition of various treatments to eliminate out resting levels of the cytokine (indicated by 0). Untreated cells (indicated by C) and BSA (BSA) are used as controls for this experiment. Cells were treated with NRG (N) for 24 hours, LPS (L) for 4 hours and ACh for 23 1/2 hours. LPS addition resulted in an increase in the concentration of TNF-α (361.34±179.17 pg/mL) compared to untreated cells (13.98±1.82 pg/mL) at 240 minutes (n=2).The concentration of TNF-α remained unchanged regardless of NRG addition in LPS treated cells (371.35±213.6 pg/mL, n=2). The addition of ACh to cells treated with NRG did not affect the concentration levels of TNF-α (308.56±43.79 pg/mL) when stimulated with LPS compared to cells that did not receive ACh (371.35±213.6 pg/mL) at 240 minutes (n=2). Error bars represent standard deviation.



	C	N	L	N+L	A+N	A+N+L
0	32.365	36.95	43.2	28.87	31.14	16.25
10	10.79	10.66	11.57	11.765	11.515	9.08
240	13.98	17.75	361.34	371.345	13.495	308.575

Figure 3.10 TNF-α expression in primary microglia are unaffected by NRG addition even in the presence of acetylcholine. All values were calculated using the slope of a standard curve of TNF-α (Figure A.2). Basal levels of TNF-α were taken before the addition of various treatments to eliminate out resting levels of the cytokine (indicated by 0). Untreated cells (indicated by C) and BSA (BSA) are used as controls for this experiment. Cells were treated with NRG (N) for 24 hours, LPS (L) for 4 hours and ACh for 23 1/2 hours. LPS addition resulted in an increase in the concentration of TNF-α (586.04±17.72 pg/mL) compared to untreated cells (7.03±1.33 pg/mL) at 240 minutes (n=2). NRG addition did not affect the concentration of TNF-α in LPS treated cells (553.96±23.08 pg/mL, n=2). The addition of ACh to cells treated with NRG did not affect the concentration levels of TNF-α (547.66±9.66 pg/mL) when stimulated with LPS compared to cells that did not receive ACh (553.96±23.08 pg/mL) at 240 minutes (n=2). Error bars represent standard deviation.



	C	N	L	N+L	A+N	A+N+L
0	8.775	7.435	9.15	7.145	7.795	11.9
10	5.915	6.9	6.725	6.03	7.575	9.33
240	7.03	10.47	586.04	553.96	10.355	547.66

Chapter 4 Discussion

The original objective of this study was to investigate a possible role for neuregulin in regulating the anti-inflammatory response. I originally hypothesized that NRG, through its binding to the ErbB receptors, might increase transcription of α7AChR in microglia. This would be an interesting result as previous work indicates that increases in α7AChR expression can lead to a decrease in TNF-α, a known stimulator of inflammation (Wang et al., 2003; De Simone et al., 2005). Based on this I further hypothesized that by adding NRG to microglial cells prior to stimulation with a mitogen I should see a reduction in the amount of TNF-α secreted by microglia upon activation. In my thesis, I have documented differences in ErbB expression in primary and immortalized cell lines. Using these cell cultures, I have further shown that NRG induces the phosphotyrosylation of the ErbB receptors and that NRG does have an effect on α7AChR expression. Yet despite being able to increase α7AChR, TNF-α expression remains unchanged in NRG treated cells.

RAW 264.7 cells where used throughout experiments as another control. Since most research done on the anti-inflammatory pathway focuses on the PNS, RAW cells were used as a representative macrophage cell line. I used RAW cells to ensure that experiments were run correctly and that the effects observed in RAW cells could also be observed in microglia.

4. 1 ErbB2-4 are expressed in primary microglia and ErbB4 is expressed in immortalized microglia and macrophages

Immortalized cell lines and primary cells appear to have distinct ErbB expression patterns. Here I have shown that immortalized microglia and macrophage cell lines express ErbB4 (Figure 3.4) while primary microglia express ErbB2-4 receptors (Figure 3.3).

Although no research has directly investigated ErbB expression in BV-2, RAW or EOC-20 cell lines, a similar immortalized cell line, N9, has been used to investigate ErbB expression. Dimayuga and colleagues (2003) show that N9 murine microglial cells express ErbB2-4 and are affected by NRG-1 addition. They were able to show that the addition of NRG-1 was able to reduce the amount of nitric oxide released by microglia in response to an assault (LPS). Nitric oxide release has been shown to trigger the expression of pro-inflammatory cytokines such as TNF-α. Therefore it is possible that NRG may also reduce TNF-α produced by microglia. I have shown that in BV-2 and EOC-20 cell lines only ErbB4 receptors are expressed (Figure 3.4). Differences between my results and the ones obtained by Dimayuga and colleagues (2003) can possibly be attributed to the age at which the cells were harvested, the methods associated with harvesting and the subtype of microglia harvested. Since two phenotypes exist for microglia, amoeboid and ramified, it is possible that this explains the difference in results. Ramified microglia appear around P5 (Ling and Wong, 1993) and continue to develop throughout the maturing CNS while amoeboid microglia are derived from blood

born monocytes and migrate into the CNS (Rezaie and Male, 2002; Wu et al., 1997). Amoeboid microglia differentiate into an intermediate form that eventually turns into ramified microglia (Rezaie and Male, 2002). Amoeboid and ramified microglia often differ in their expression of receptors and phagocytotic activity (Wu et al., 1997; Ling and Wong, 1993). Depending on the age at which N9 cells were originally harvested and the specific type of microglia cultured (amoeboid or ramified) could account for these differences. A comparison of morphologies between the cell cultures using phase microscopy could confirm this hypothesis.

I have shown that primary microglia express ErbB2-4 receptors (Figure 3.3). This result is of particular interest since these cells express an additional two receptors not shown in immortalized cells lines and differ from results described from the literature (Dimayuga et al., 2002; Gerecke et al., 2001; Chaudhury et al., 2003; Cannella et al., 1999)(Figure 3.4 and table 2). Current literature suggests that ErbB2 expression in microglia is confined to tissues that are diseased or damaged (Table 2; Chaudhury et al., 2003; Canella et al., 1999). This is a possible explanation as to why ErbB2 expression is seen in my primary microglia cultures and not in my immortalized cells. Since microglia do phagocytose surrounding debris during damage combined with the fact that both neurons and astrocytes do express ErbB2 receptors there is a possibility that these cells are the source of the ErbB2 receptors found in primary microglia (Figure 3.3)(Sharif et al., 2009; Chaudhury et al., 2003; Gerecke et al., 2001). Before cells were purified, microglial cells were grown up in culture with astrocytes which do express ErbB2-3 (Sharif et al., 2009). Often during purification, cells are damaged and often harvested multiple times before a culture becomes pure. The steps required for purification

encourage microglia to phagocytose astrocytes. Microglial cells were grown in isolation for 24 hours before experimentation so it is possible that any receptors engulfed would show during analysis because microglial cells would not have had enough time to degrade the components of the engulfed ErbB receptors.

Phagocytosis poses similar problems in determining the origins of ErbB3 receptors. Gerecke and colleagues (2001) along with Canella and colleagues (1999) support the findings that microglia express ErbB3 receptors. I have also shown that primary microglia do express ErbB3 receptors (Figure 3.3). However, again it is possible that this result is due to phagocytosis over actual microglial expression.

Finally, there are differing reports concerning the origins of receptors in microglial cells (Chaudhury et al., 2003; Gerecke et al., 2001; Erlich et al., 2002; Canella et al., 1999). ErbB4 expression levels are upregulated at the site of injury and disease and confocal microscopy has confirmed that some ErbB4 expression is due to the phagocytosis of neuronal cells (Erlich et al., 2002). However, since neuronal cells were eliminated from my samples at postnatal day one and were cultured at 12-14 days *in vivo,* the likelihood that engulfed neuronal ErbB4 receptors would survive degradation is highly unlikely. Engulfment of astrocytes by microglia is also unlikely since cortical astrocytes do not possess ErbB4 receptors therefore expression of the ErbB4 receptors in microglia are not due to engulfment of astrocytes. Astrocytes obtained from the hypothalamus do contain ErbB4 receptors but are in such a small percentage compared to cortical astrocytes that their influence is likely negligible (Sharif et al., 2009). It can then be concluded that primary microglia, like their immortalized counterparts, do possess ErbB4 receptors.

A likely explanation to account for the differences in ErbB receptor expression could be due to the specific regions where microglia are harvested from. This could be due to differing ErbB expression in microglia attributed to differences in function or due to phagocytosis of other cells found within those specific regions. Gerecke and colleagues (2001) identified varying populations of ErbB receptors dependant on location of isolation and specific cell type (Gerecke et al., 2001). This could mean that ErbB receptor expression in microglia could depend on where the cells are harvest from in the brain or what population of cells surrounds the microglia at time of harvest. Alongside the results obtained from Sharif and colleagues (2009) where astrocyte ErbB expression is dependent on location, differences in microglia expression in the brain could be attributed to the region at which the cells were harvested as previously mentioned or due to the populations of cells surrounding microglia and those cells specific ErbB expression.

It can be concluded that there is at least ErbB4 expression in microglia with the possibility of ErbB2 and ErbB3 expression in primary microglia. Future investigations should use confocal microscopy to determine if ErbB2 and ErbB3 expression in microglia was due to phagocytosis. Additionally, primary cells could be harvested at a later date after purification. Having shown that microglia possess ErbB4 receptors, the next step was to investigate if these receptors would become phosphorylated with NRG addition.

4.2 Neuregulin phosphorylates ErbB4 in microglia and macrophages

Through the use of anti-phosphotyrosine antibodies, I was able to demonstrate that microglial ErbB receptors are phosphorylated in response to NRG (Figure 3.4). Tyrosine phosphorylation in microglia is important for cell activation (Karpet et al., 1994) and can be achieved through the binding of NRG to the ErbB receptors (Buonanno and Fischbach, 2001). More specifically, ErbB4 receptors in microglia and macrophage cell lines were phosphorylated with NRG addition (Figure 3.4). It is not surprising that I saw no difference in ErbB2 and ErbB3 receptor phosphorylation with NRG addition as these receptors are not expressed in immortalized cell lines (Figure 3.4).

Due to the limited supply of primary cells, phosphotyrosylation could not be investigated in primary cells. These experiments could help to rule out the ambiguities regarding ErbB2 and ErbB3 expression in primary cells. ErbB2 or ErbB3 phosphorylation with NRG addition, would allow us to conclude that the ErbB receptors shown in figure 3.3 were in fact expressed on the cell surface of microglia and not derived from the phagocytosis of astrocytes' receptors.

4.3 Neuregulin affects the expression of α7AChR in microglia

I have shown that ErbB4 receptors in my microglial cultures become phosphorylated with the addition of neuregulin (Figure 3.4). Further, I was able to show that NRG addition leads to an increase in α7AChR over untreated cells in BV-2 and primary cells (Figures 3.5 and 3.6). Since ErbB4 is the only ErbB receptor present in BV-

2 cells, it potentially forms homodimers with these initiating a signaling cascade leading to increases in α7AChR expression.

Primary cells show a similar result to that of BV-2 cells. Neuregulin treatments increase α7AChR expression (Figure 3.6) compared to untreated. However, unlike BV-2 cells, the untreated primary microglial cells have an undetectable level of α7AChR. This is perhaps simply a reflection of the amount of material available for loading onto the gel.

As mentioned previously, the addition of ACh to NRG and NRG + LPS treatments provides a closer environment to that which would be found *in vivo.* The anti-inflammatory pathway begins with an assault (LPS) which eventually stimulates the release of TNF-α in microglia (de Jonge and Ulloa, 2007; Ulloa, 2005). When it is time to decrease or eliminate inflammation, the vagus nerve releases ACh to then bind to α7AChR thereby decreasing TNF-α release (De Simone et al., 2005; Wang et al., 2003; de Jonge and Ulloa, 2007; Ulloa, 2005**).** When ACh is added to NRG treated cells there is an increase in α7AChR expression when LPS is added versus LPS untreated (Figure 3.6).

Due to the arduous process of culturing primary cells combined with their extremely finite populations, α7AChR experimentation was carried out once. Future experiments could replicate this immunoblot and provide a more statistical and definite answer as to NRG affects on α7AChR expression in primary cells.

4.4 Neuregulin does not affect TNF-α secretion in microglia or macrophages

In figures 3.5 and 3.6 I have shown that the addition of NRG results in increased α7AChR expression. Taking De Simone and colleagues (2005) results into account one

might have expected a reduction in TNF-α concentration when microglia were treated with NRG and ACh compared to cultures left untreated (De Simone et al., 2005). Instead, what was seen was no difference in TNF-α concentration in any NRG treated sample (Figures 3.8 and 3.9) despite the addition of ACh. Therefore, contrary to what was originally hypothesized, an increase of α7AChR did not relate to a decrease in TNF- α concentration at least under these conditions.

Possible explanations as to why TNF-α was unaffected by NRG could be due do the saturation of ACh and its receptor. Additionally, the incubation time of LPS before media extraction could have also resulted in differences in TNF-α concentration. ACh is one of the ligands used to activate α7AChR and is normally released by the vagus nerve upon stimulation (De Simone et al., 2005; Wang et al., 2003; de Jonge and Ulloa, 2007**).** It is possible that the amount of ACh added was insufficient to mediate the anti-inflammatory properties of α7AChR. Repeat experiments could be performed using varying concentrations of ACh and measuring its effect on TNF-α. Nicotine could also be used in place of ACh since it is also a ligand for α7AChR but binds more strongly to the receptor (de Jonge and Ulloa, 2007; Ulloa, 2005**).**

Varying the LPS treatment incubation time for the ELISA could account for the difference in TNF-α concentration. In my ELISA experiments, cells were treated with LPS for four hours prior to media extraction. In both rat and mouse systems, four hours seems to be the desired incubation time to allow for peak TNF-α concentrations but expression has also been seen twenty four hours after addition (De Simone et al., 2005). Analyzing the incubation times of LPS prior to media extraction could result in a more

desirable incubation time. It is possible that NRG mediated effects require longer incubation with LPS to get a reduction in TNF-α levels.

4.5 Conclusions

There are many differences that exist between using immortalized and primary cell cultures. Here I have established that differences do exist in microglial cell lines with respect to ErbB receptor expression (Figure 3.3 and Figure 3.4). Immortalized microglia posses ErbB4 receptors while primary microglia posses ErbB2-4 receptors.

Neuregulin is able to increase α7AChR expression in microglia (Figure 3.5). NRG mediates these effects by binding to ErbB4 homodimers which then become phosphorylated (Figure 3.4) Despite NRG's ability to affect α7AChRs it is unable to change the concentration of TNF-α (Figure 3.8 and Figure 3.9). In conclusion, even though NRG is able to increase α7AChRs it is unable to mediate the anti-inflammatory pathway.

4.6 Future directions

As mentioned previously, primary cell cultures and time were the limiting factors in this investigation. With more of both the evidence supporting NRG's ability to increase α7AChR expression, in primary cells would be stronger. Additionally, phosphorylation and confocal studies on primary cells would help to explain the origins of ErbB2 and ErbB3 receptors in primary cells. If by using microscopy the ErbB

receptors were localized to lysosomes then the presence of these additional two receptors might be attributed to the phagocytosis of astrocytes. Another possible way to rule out astrocytes hindrance is to culture the cells longer once purity is obtained but this is not easily achieved. Since microglia need to reach a specific confluence with astrocytes before they can be cultured on their own, early separation could result in microglia populations that do not grow. Also, due to the finite life span of primary cell cultures, once purity is reached there is no predetermined amount of time that microglia will live for. Having microglia grow for a few more days alone in culture would be an easy way to eliminate out contamination if they survive. Possible transfection of primary microglia with a virus could result in extended cell survival as seen in EOC-20, BV-2 and N9 immortalized cell lines.

It would be interesting to investigate NRG effects on IL-12, an anti-inflammatory molecule released by microglia. Takahashi and colleagues (2006) have shown that using α7AChR antagonists inhibited the production of IL-12 (Takahashi et al., 2006). Since NRG was shown to increase α7AChR in microglia (Figure 3.5) it is possible that it could also regulate the amount of IL-12 released. Similar experiments used for TNF-α could therefore be used for IL-12 (ELISA).

Being able to regulate the production of pro-inflammatory cytokines in microglia through a mechanism dependant on α7AChRs would be beneficial in a number of pharmaceutical applications. In particular, dysregulated microglia are believed to play an important role in neurodegenerative diseases such as Alzheimer's disease (AD) (Streit et al., 2004; Raivich and Banati, 2004; Chaudhury et al., 2003). Alzheimer`s disease is associated by uncontrolled inflammation, gliosis, neuritic plaques and neuronal death

(Chaudhury et al., 2003). Chaudhury and colleagues (2003) found activated microglia with increased ErbB4 expression in the neuritic plaques of AD patients (Chaudhury et al., 2003). In addition to increased ErbB expression, they also found an upregulation of NRG-1 in neuritic plaques (Chaudhury et al., 2003). The activation of microglia leading to the overexpression of TNF-α also plays a critical role in the pathology of AD (Streit et al., 2004). If NRG were able to selectively regulate the amount of TNF-α released by microglia it is possible that NRG addition would alleviate some of the damage caused by uncontrolled inflammation. In neuritic plaques there is an increase in NRG expression therefore it is possible that NRG plays a role in neuroprotection. Dimayuga and colleagues (2003) reported that NRG did increase cell viability so it is also possible that NRG addition could aid in neurodegenerative disorders. The mechanisms are still unknown, but since I was able to show that NRG is able to increase α7AChR levels it is possible that NRG still plays a role in neuroprotection. Further studies could look at the role of NRG and microglia in disease and what possible therapies NRG treatments could provide.

Appendix A

Figure A.1 Standard Curve of mouse TNF-α concentration used for Figures 3.7 and 3.8. Calibration curve was constructed by performing two fold serial dilutions of TNF-α with a starting concentration of 1000 pg/mL. Slope was used to determine the concentration of TNF-α secreted by RAW (Figure 3.7) and BV-2 (Figure 3.8) cells.

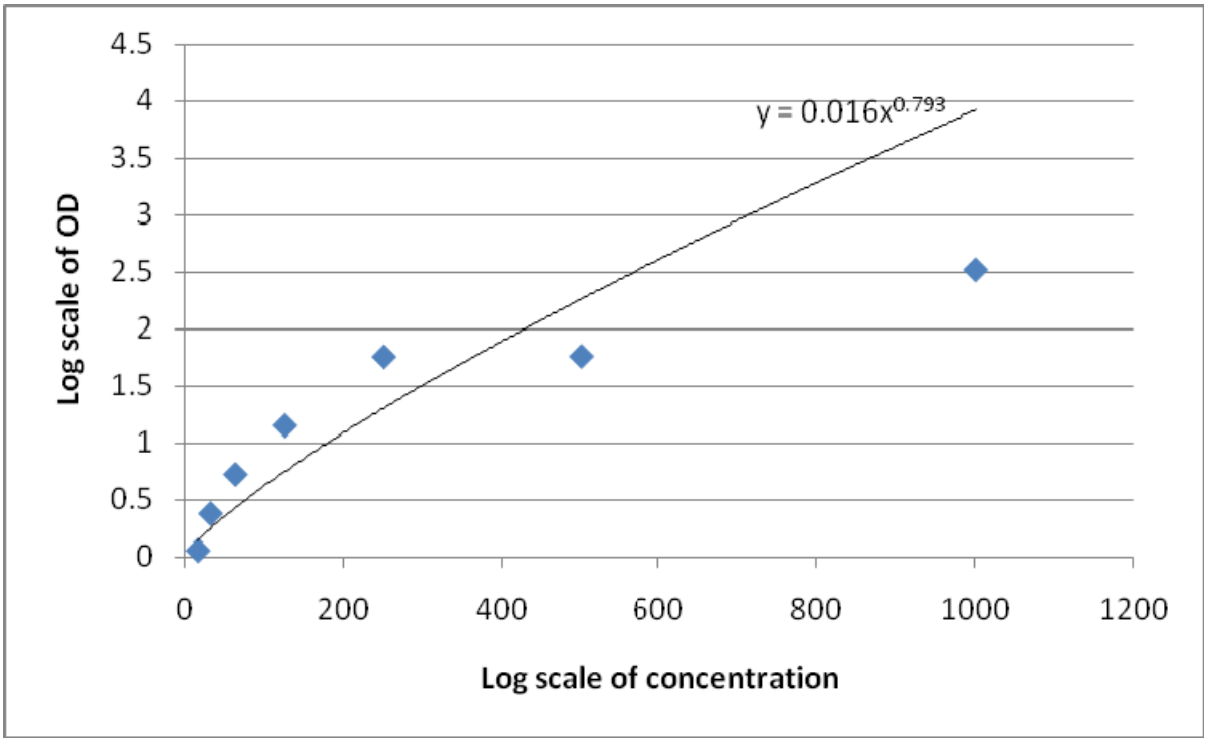
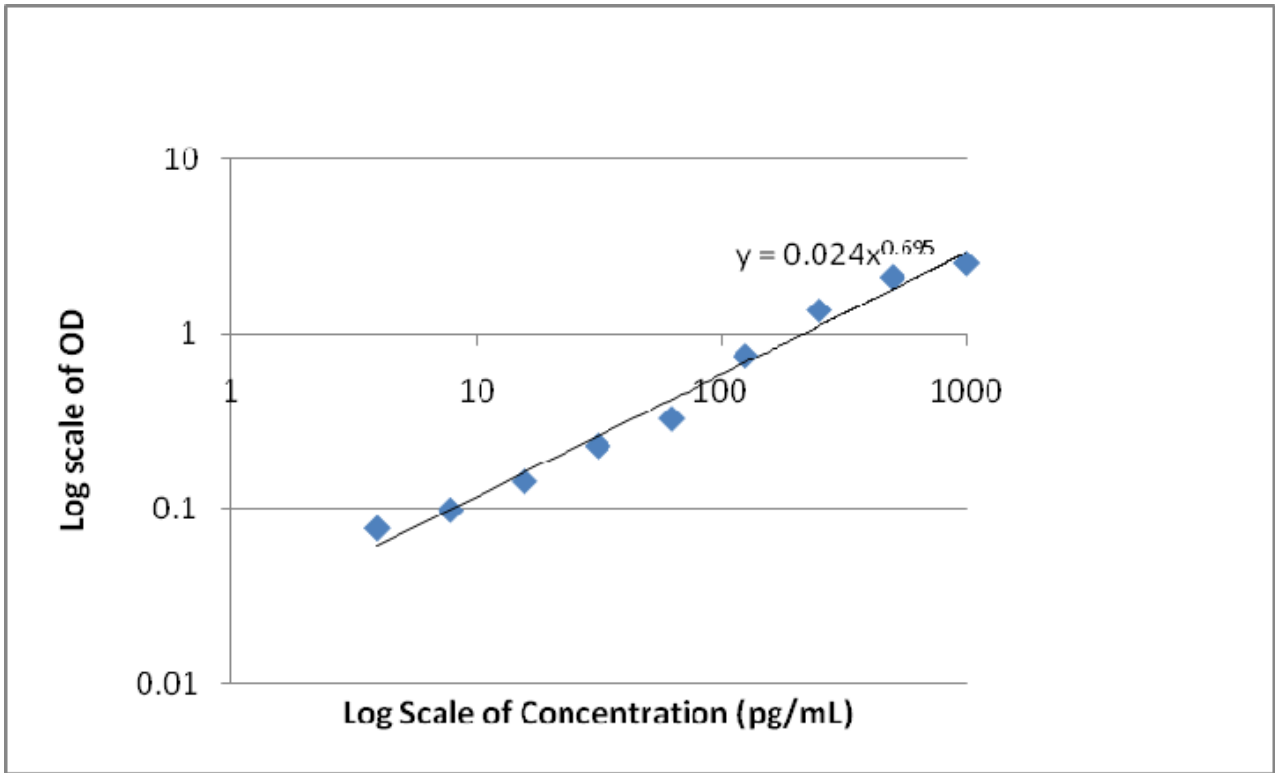

y = 0.016x^0.793
Log scale of OD
Log scale of concentration
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
200
400
600
800
1000
1200

Figure A.2 Standard Curve of mouse TNF-α concentration used for Figures 3.9 and 3.10. Calibration curve was constructed by performing two fold serial dilutions of TNF-α with a starting concentration of 1000 pg/mL. Slope was used to determine the concentration of TNF-α secreted by BV-2 (Figure 3.9) and primary (Figure 3.10) cells.


10
1
0.1
0.01
1
10
100
1000
y = 0.024x^0.695
Log scale of OD
Log Scale of Concentration (pg/mL)

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