

Potential Roles for the Neurotrophic Molecules Agrin and Neuregulin in Regulating Aspects of the Inflammatory Response

by

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A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Doctor of Philosophy
in
Biology

Waterloo, Ontario, Canada, 2014

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis,
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Abstract

Agrin and neuregulin are neurotrophic molecules well known for their roles at synapses in the peripheral and central nervous systems. The expression of these two molecules is not restricted to these sites however, as they are broadly expressed across multiple organ systems. What roles do agrin and neuregulin play within these alternate systems; what is the function of these molecules outside the nervous system? Here I investigate potential roles for agrin and neuregulin in inflammation. Inflammation is an immediate response by innate immune cells, primarily macrophages, to infection and is characterized by the synthesis of pro-inflammatory mediators. The innate immune system possesses multiple redundant mechanisms to locally control inflammation. The neuro-immune axis is one means of control. Often called the cholinergic anti-inflammatory pathway, it acts to regulate local inflammation via nerve-secreted acetylcholine signaling through the homopentameric $\alpha 7$ nicotinic acetylcholine receptors ($\alpha 7\text{nAChR}$) present on macrophages.

Both agrin and neuregulin have been independently described to share an intricate relationship with acetylcholine receptors (AChR) in the nervous system. Agrin is best known for its role in AChR aggregation at the neuromuscular junction while neuregulin has related roles in AChR transcription, cell survival, communication and differentiation. Based on the common characteristics of synapses in the nervous and immune systems we were curious to see if agrin and neuregulin played analogous roles on macrophages.

Here we show that agrin and its receptor dystroglycan are expressed on RAW264.7 macrophages. In addition, agrin treated macrophages demonstrate increased endogenous agrin and $\alpha 7\text{nAChR}$ expression. By blocking α -dystroglycan (α -DG), a receptor for agrin, with an anti- α -DG antibody we further saw a reduction in agrin expression. We also show that agrin is able to aggregate surface $\alpha 7\text{nAChRs}$ and transmembrane agrin co-localizes with $\alpha 7\text{nAChRs}$ therein. Agrin appears to induce approximately a 15-fold increase in anti-inflammatory cytokine IL-10 in macrophages but does not increase pro-inflammatory

cytokine TNF- α or IL-6 synthesis. Agrin-treated macrophages challenged with LPS, a potent activator of inflammation, exhibit a 57% decrease in IL-6. Macrophages treated with agrin also exhibit a 4-fold increase in STAT3, a regulator of anti-inflammatory action.

The potential anti-inflammatory effects of agrin in the periphery parallel previous work describing the effects of neuregulin in the brain. Previous work completed by our lab suggests a role for neuregulin in augmenting the expression of $\alpha 7nAChRs$ on microglia, the macrophages of the brain, but not in peripheral macrophages. Here we show that treatment of LPS challenged microglia with neuregulin produces an 88% decrease in IL-6 and a 33% decrease in TNF- α . These results indicate both agrin and neuregulin are able to induce an increase in $\alpha 7nAChRs$ and augment the synthesis of pro- and anti-inflammatory cytokines in their respective systems. These results also further the support the evidence of neuro-immune crosstalk in the immune system. Taken together these results present two novel players in inflammatory regulation by macrophages in the periphery and CNS.

Acknowledgements

I would like to begin by thanking my supervisor Chris Jacobson, my co-supervisor Bernie Duncker, and the members of my committee: Mungo Marsden and Matt Vijayan, for supporting me through thick and thin, and for your unequivocal faith in me.

To my family and friends, who have been supportive and patient when I claimed that “I will be done... soon” for a very long time.

To my partner, the last few years would have not been possible without your support.

Thank you, all.

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List of Abbreviations

α 3NKA	alpha3 subunit of $\text{Na}^+ \text{K}^+$ ATPase channel
α 7nAChR	alpha7 nictonic acetylcholine receptor
α BTX	alpha bungarotoxin
ACh	acetylcholine
AChE	acetylcholinesterase
AChR	nicotinic acetylcholine receptor
Akt	protein kinase B
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APC	adenomatous polypsis coli
ARIA	acetylcholine receptor-inducing activity
ATCC	American tissue culture
BBB	blood brain barrier
BSA	bovine serum albumin
Cdc42	cell division control protein 42
Cdk5	cyclin-dependent kinase 5
CHO	Chinese hamster ovary
CNS	central nervous system
CREB	cAMP response element binding protein
DAG	dystrophin-associated glycoproteins
DAPC	dystrophin-associated protein complex
DG	dystroglycan- α/β
DMEM	Dulbecco's modified minimal essential media
Dok7	docking protein 7
Dvl	dishevelled
ECM	extracellular matrix

EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK	extracellular signal regulated kinase
FBS	fetal bovine serum
Fyn	proto-oncogene tyrosine-protein kinase
GABA	gamma-aminobutyric acid
GABP	GA-binding protein
GAG	glycosaminoglycan
GGF2	glial growth factor 2
GluR	glutamate receptor
GlyR	glycine receptor
Grb2	growth factor receptor-bound protein 2
GSK3	glycogen synthase kinase 3
HRP	horseradish peroxidase
HS	horse serum
Hsp40	heat-shock protein 40
Hsp70	heat-shock protein 70
Hsp90 β	heat-shock protein 90 beta
IL-1 β	interleukin-1beta
IL-10	interleukin-10
IL-6	interleukin-6
IL-8	interleukin-8
Jak2	Janus kinase 2
JNK	c-jun N-terminal kinase
KO	knock out
LPS	lipopolysaccharide
LRP4	low-density lipoprotein receptor-related protein 4
MAPK	mitogen-activated protein kinase

MEK	mitogen-activated protein kinase kinase
MHC	multihistocompatibility complex
MMP	matrix metalloproteinases
MuSK	muscle-specific kinase
NDF	neu differentiating factor
NFDM	non-fat dehydrated milk
NF-κB	nuclear factor kappa B
NMDA	N-methyl-D-aspartic acid
NMJ	neuromuscular junction
NRG	neuregulin 1
NtA	N-terminal agrin sequence
PAK	p21-activated kinase
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PI3K	phosphatidylinisitol-3 kinase
PNS	peripheral nervous system
PSD-95	post-synaptic density 95
RhoA	Ras homolog gene family member A
SMDF	sensory- and motor-neuron derived factor
STAT3	signal transducer and activator of transcription 3
TBS	Tris-buffered saline
Tid1	tumorous imaginal discs
TLR4	toll-like receptor 4
Tm	transmembrane
TNF-α	tumor necrosis factor alpha

Chapter 1

Introduction

1.1 General introduction

The word *synapse* is derived from the Greek root ‘*sunaptein*’ meaning to fasten together (Yamada and Nelson, 2007). Synapse formation, also called synaptogenesis, is pivotal for cell-to-cell communication and is typically dependent on the clustering and activation of surface receptors and the establishment of efficient signal transduction. Traditionally the study of synapses involved neurons and the membrane-to-membrane junctions they formed with other neurons or target cells. However, a more modern interpretation of synapses has them defined as a point of cell-cell contact or interaction between a variety of cells and tissue types. A modern synapse is thus a stable connection or junction that forms between homologous or heterologous cells for cell-to-cell communication (Dustin and Colman, 2002). Most synapses can be categorized into electrical or chemical synapses, and are distinguished by their mode of transmitting signals. Electrical synapses, those that communicate via gap junctions, are found in a minority of neuronal synapses and typically only in the mammalian inferior olfactory nucleus, neocortex, hippocampus and thalamus (Eccles, 1982; Bennett, 1997; Connors and Long, 2004). The vast majority of neuronal synapses then are chemical synapses and cell-to-cell communication is via chemical intermediaries such as neurotransmitters.

Synapses found outside of the nervous system are similar to those within and are characterized by stable adhesions, the secretion of chemical factors, as well as the clustering of various pre-synaptic and post-synaptic molecules. The formation of a functional synapse is

reliant on multiple factors that regulate, organize and reorganize membrane proteins, lipids and receptors at the site of cellular contact. This process is mediated in a sequential manner by a number of proteins, and is essential for effective and efficient cell-cell signalling (Reviewed in Yamada and Nelson, 2007). Synapses can form between a variety of cell and tissue types, including motor neurons and muscle (termed a neuromuscular junction (NMJ) or post-synaptic apparatus), between neurons (interneuronal synapses), and between immune cells (immune synapses) (Figure 1). Though these synapses are extrinsically different due to the cells involved, structurally and functionally they are intrinsically similar in that they contain stable contacts mediated by cellular adhesion molecules allowing cell-cell communication to be achieved by the secretion of chemical mediators that then bind to their specific receptors.

Agrin and neuregulin are two molecules with very prominent roles in synapse formation. They share a number of common functions and signaling mechanisms in both the peripheral and central nervous systems and appear cooperative in processes mediating acetylcholine receptor (AChR) clustering, stability, density and regulation (Hoch, 1999).

AChRs are ubiquitously expressed across many tissues and cells where they have been implicated in a variety of regulatory roles. Of particular importance to this thesis is the expression of AChRs on endothelial and immune cells, such as macrophages. AChRs on these cells have been described to be a central part of a neuro-immune axis, termed the cholinergic anti-inflammatory pathway, crucial for regulating inflammation.

From an evolutionary standpoint, one might expect the conservation of molecules and mechanisms throughout synaptic contacts in a variety of tissues. Given their pivotal role in the formation and maintenance of synapses within the nervous system, agrin and neuregulin are likely key players in cell-to-cell contacts fitting our more modern, broader definition of a synapse. Based on the close relationship between the organizing molecules of neural synaptogenesis with AChRs, and the abundance of AChRs in non-neural tissues, the study of these molecules and their expression and roles in other tissues, becomes very attractive. Here I introduce agrin and neuregulin, including their intricate relationship with AChRs, and investigate their potential roles in modulating inflammation and the cholinergic anti-inflammatory pathway.

1.2 Agrin

1.2.1 Agrin organizes synapses

The protein agrin is a highly glycosylated heparin sulfate proteoglycan that is broadly expressed as multiple isoforms in the basement membranes and on cell surfaces in a number of tissues (Tsen et al., 1995a). Agrin, from the Greek word ‘*agrein*’ meaning to assemble, was originally named for its notable ability to aggregate AChRs (Nitkin, 1987). Though originally isolated in the electric organ of *Torpedo californica* (Nitkin, 1987; Smith et al., 1987), agrin has been extensively described at the mammalian NMJ where it plays a pivotal role in synapse formation (Rupp et al., 1992; Fallon and Hall, 1994; Gautam et al., 1996) by

regulating cell-cell interactions (Cole and Hafner, 1996). Synapse formation at the NMJ is primarily characterized by the aggregation and stabilization of AChRs (Reviewed in Sanes and Lichtman, 1999 and Kummer et al., 2006), an essential process required for efficient nerve-myofibre communication and ultimately muscle function.

The core agrin protein is approximately 200kDa but when glycosylated this protein can reach a molecular weight of up to 600kDa (Tsen et al., 1995a). It is composed of multiple domains and has a linear sequence of ~1900 amino acids (Rupp et al., 1991). Agrin can be divided into 4 domains (Figure 2) (Reviewed in Iozzo, 1998). Domain I is a globular domain identified as N-terminal agrin (NtA) that binds laminin and thus becomes immobilized in basal laminas (Denzer et al., 1997). Domain II is composed of nine follistatin-like repeats each containing protease inhibitor type domains, a cystine/proline-rich domain and a laminin III domain (Rupp et al., 1991). It also contains N-glycosylation sites and glycosaminoglycan (GAG) attachment sites. Domain III contains a central SEA motif, serine/threonine-rich domains, GAG attachment sites and is highly glycosylated (Rupp et al., 1991). The final region, domain IV, contains a serine/threonine-rich domain, four epidermal growth factor (EGF)-like repeats, four laminin-like G domains, and three splice sites X, Y and Z (Rupp et al., 1991; Ferns et al., 1992). It is within this final region that the laminin-like G domains confer agrin's binding to its receptors. Thus, the majority of the C-terminal half of agrin is particularly important for binding (laminin-like G domains) and clustering activity (Bowe et

al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994; Gesemann et al., 1996).

1.2.2 Alternative splicing mediates agrin function

Alternative mRNA splicing at several sites within the agrin transcript produces a number of agrin protein isoforms. These events occur at one site in the N-terminus and three sites, the X, Y and Z sites, in the C-terminus. The agrin gene contains two different promoters that allow for tissue-specific regulation and can produce two isoforms that differ in their N-termini: the short-N-terminus (SN) and the long-N-terminus (LN). These two isoforms differ by a sequence of 49 (SN) or 150 amino acids (LN) (Burgess et al., 2000; Neumann et al., 2001). SN-agrin gives rise to transmembrane agrin (Tm-agrin), which is highly expressed in the central nervous system (CNS), and is potentially involved in cell adhesion processes (Tsien et al., 1995b). Tm-agrin does not contain NtA, does not bind laminin and can induce AChR clustering, however it does not appear to play a role in NMJ formation (Neumann et al., 2001). LN-agrin contains the NtA domain and signal peptide and is essential to synapse formation at the NMJ. Furthermore, LN-agrin is preferentially expressed in non-neuronal cells and neurons that innervate non-neuronal cells such as motor neurons (Stetefeld et al., 2001).

Further agrin heterogeneity is generated through alternative splicing at its C-terminal end (Ferns et al., 1992) with these isoforms differing greatly in function (Campanelli et al.,

1996). Agrin's ability to aggregate AChRs (Ferns et al., 1992; 1993; Campanelli et al., 1996) as well as its ability to bind its receptors (Gesemann et al., 1996) is dependent on splicing at the Y and Z sites (Figure 2). The isoform expressed by muscle, *muscle agrin*, has either a four amino acid insert at the Y site or no inserts at the Y and Z sites. Muscle agrin exhibits low AChR clustering capabilities even at high concentrations (Godfrey, 1991; Lieth et al., 1992; Rüegg et al., 1992; Hoch et al., 1993). In motor neurons, the inclusion of an eight amino acid insert at the Z position produces *neural agrin* (Ferns et al., 1992). It is this isoform, neural agrin, that is responsible for agrin's activity at the NMJ (Reist et al., 1992). Splicing at this position does not appear to occur in peripheral tissues outside of the nervous system (Khan et al., 2001) and the function of these broadly expressed non-neuronal agrin isoforms is unclear.

Neural agrin, facilitates the clustering of AChRs on muscle through a complex signaling pathway that results in the redistribution of AChRs, re-organization of the actin cytoskeleton (Sanes and Lichtman, 1999), and increased transcription of AChR subunits (Jones et al., 1996). Agrin appears to have numerous binding partners on muscle or within the basal lamina surrounding muscle, including integrins, heparin, and laminins (Reviewed in Sanes et al., 1998). Two, however, are of particular interest, low-density lipoprotein-related protein 4 (LRP4; Kim et al., 2008; Zhang et al., 2008) and dystroglycan (DG; Campanelli et al., 1994; Gee et al., 1994; Sealock and Froehner, 1994) and their role in mediating agrin function will be discussed below.

1.2.3 The Agrin-MuSK signaling pathway mediates AChR clustering

Agrin activity is mediated by a heterodimeric receptor complex comprised of a muscle-specific receptor tyrosine kinase, a co-receptor and a cytosolic peripheral membrane protein. Muscle-specific kinase (MuSK), LRP4 and rapsyn, are central to AChR aggregation and are necessary for synapse formation at the NMJ. Overexpression of any of these components results in the clustering of AChRs and knockout (KO) experiments in mice targeting any of these three proteins result in impaired synapse development, and impaired nerve-induced AChR aggregation. The strongest phenotype was observed in MuSK KO mice, which exhibited no AChR aggregates (DeChiara et al., 1996). Moreover, MuSK KO mice are unresponsive to agrin, suggesting that it is MuSK that functions as the agrin receptor.

On myotubes, MuSK is rapidly phosphotyrosylated in response to agrin treatment. However, if MuSK alone is transfected into fibroblasts it does not become phosphorylated when treated with agrin indicating that MuSK must function through another component, perhaps a co-receptor (Glass et al., 1996). This unknown muscle specific co-receptor was termed MASC (for *Myotube-Associated Specific Component*) and was not identified until fairly recently as LRP4 (Kim et al., 2008; Zhang et al., 2008). Finally, rapsyn, originally called the 43 kd post-synaptic protein, or 43K, is highly concentrated in the electric organ of *Torpedo californica* and mammalian vertebrate NMJs (Porter and Froehner, 1983) and interacts with AChRs (Burden et al., 1983). KO experiments targeting rapsyn, a cytoplasmic peripheral membrane protein that colocalizes to AChRs, exhibit defective NMJs. These

indicated that rapsyn is also necessary for AChR aggregation (Gautam et al., 1996). The interactions of a variety of other agrin-associated molecules are described below.

Agrin secreted by the nerve terminal binds to the MuSK co-receptor LRP4, initiating a trans-phosphorylation event that results in AChR aggregation and cytoskeleton re-organization (Kim et al., 2008; Zhang et al., 2008). MuSK signaling is crucial for AChR aggregation events in the developing synapse (Glass et al., 1996)(Figure 3). Phosphorylation of MuSK leads to the recruitment of the adaptor proteins tumourous imaginal disc (Tid1; Linnoila et al., 2008), downstream-of-kinase or docking protein 7 (Dok7; Bergamin et al., 2010), rapsyn (Apel et al., 1997; Glass and Yancopoulos, 1997), dishevelled (Dvl) and p21-activated kinase (PAK; Luo et al., 2002). The role of these adaptor proteins and how they function with respect to MuSK activation are described below.

Dok7 is a cytoplasmic adaptor protein originally referred to as p62 (Yamanashi and Baltimore, 1997). Dok7 KO mice exhibit a similar phenotype to MuSK and Agrin KO mice and as a result appear to be essential for NMJ formation (Okada, 2006). Dok7 binding to MuSK is regulated by Tid1 (Linnoila et al., 2008) which is required for MuSK activation (Okada, 2006) and facilitates trans-phosphorylation events (Bergamin et al., 2010). Tid1 is a mammalian homolog of Drosophila tumor suppressor and heat-shock protein 40 (hsp40) and can partially activate MuSK in the absence of agrin. This allows the binding and dimerization of Dok7 (Linnoila et al., 2008). Tid1 also regulates the activity of the GTPases Rac1, RhoA, and cdc42 in an agrin-dependent manner which regulate AChR clustering and mediates

AChR phosphorylation (Linnoila et al., 2008). Tyrosine phosphorylated AChRs demonstrate an enhanced association with the cytoskeleton (Borges, 2001) via an agrin-induced increase in rapsyn (Moransard et al., 2003). Tid1 is part of the hsp40 family and binds to hsp70 (Linnoila et al., 2008), which interacts with rapsyn via hsp90 β (Luo et al., 2008). Hsp90 β thus regulates rapsyn turnover and maintains the stability of rapsyn associated with AChRs (Luo et al., 2008). In addition to AChRs and hsp90 β , rapsyn also interacts with β -catenin/ α -catenin which is also linked to the cytoskeleton (Zhang et al., 2007) and stabilizes AChRs by inhibiting calpain activity in a calcium-dependent manner (Chen et al., 2007). This supports early findings that indicated that agrin mediates AChR clustering in a calcium-dependent manner (Megeath and Fallon, 1998; Megeath et al., 2003). Therefore, agrin-MuSK signaling is heavily reliant upon the function of Tid1 and its associated molecules, through which stability and clustering can be achieved.

Another MuSK binding partner, Dvl was originally discovered in *Drosophila* where it is involved in the development of arrays of polarized cells (Perrimon and Mahowald, 1987). It appears that Dvl and PAK also mediate agrin-induced AChR clustering via activated Rho GTPases Rac1 and cdc42 signaling mediated by Dvl bound to MuSK and PAK (Weston et al., 2000; Luo et al., 2002). This mechanism is not unlike the Wnt pathways which have also been implicated in NMJ development (Reviewed in Henríquez and Salinas, 2012). In particular Wnt3 and agrin activate complementary pathways that activate the Rac and Rho GTPases, respectively, both of which, as described earlier, mediate AChR phosphorylation

and clustering (Henríquez et al., 2008). Adenomatous polyposis coli (APC), a known effector of Wnt signaling, is also associated with AChRs and appears necessary for clustering and the regulation β-catenin stability (Wang et al., 2003b). Though a variety of molecules have been implicated in complementing the clustering of AChRs through association with MuSK, it is evident that this process is complex and converges upon only a few common signaling mediators, namely Rac and cdc42.

Interestingly, ACh and agrin appear to have antagonistic effects at synapses. ACh secreted by the nerve terminal binds to the AChRs present on muscle inducing activity. Cholinergic signaling stimulates an increase in calpain activity which activates cyclin-dependent kinase 5 (Cdk5; Chen et al., 2007) destabilizing AChR clusters (Lin et al., 2005). In contrast it appears that agrin signaling enhances the association of rapsyn with calpain, decreasing its activity and thus inhibiting AChR cluster dispersal (Chen et al., 2007).

1.2.4 Agrin also signals through DG complexes

DGs are dystrophin-associated glycoproteins (DAGs) that are highly concentrated at NMJs (Ohlendieck et al., 1991) and have been implicated in synapse formation (Reviewed in Winder, 2001 and Pilgram et al., 2010). DGs are composed of α-DG, a peripheral membrane protein that is associated non-covalently with β-DG, an integral membrane protein, both of which are the product of the post-translational cleavage of a single transcript (Ibraghimov-Beskrovnyaya et al., 1992; Henry and Campbell, 1996). DGs are a component of a dystrophin-

associated protein complex (DAPC) and are necessary for the stabilization and condensation of AChRs at the NMJ through their interaction with the underlying cytoskeleton and overlying extracellular matrix (ECM; Ervasti and Campbell, 1991; Carbonetto et al., 1999; Jacobson et al., 2001) (Figure 4). DG is an integral part of the DAPC and is absent in the muscle of Duchene's muscular dystrophy (Ervasti et al., 1990). Agrin binds to α -DG with high affinity (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994) however the agrin domain necessary for this binding is not necessary for agrin-MuSK signaling (Meier et al., 1996; Jacobson et al., 1998). As such it does not appear to be a co-receptor with MuSK (Jacobson et al., 1998) or by extension with LRP4. However further studies have demonstrated undeniably that DG is important in the process of AChR aggregation (Jacobson et al., 1998; Kahl and Campanelli, 2003) though the exact mechanism is still unclear.

DG reportedly has extracellular mechanisms of regulating aspects of AChR clustering primarily through α -DG and functions to mediate the size and density of AChR clusters (Jacobson et al., 2001). Cells deficient in α -DG exhibit a decrease in AChR clusters (Jacobson et al., 1998) perhaps due to the intact juxtamembrane of β -DG, which appears to inhibit agrin activity (Kahl and Campanelli, 2003). Thus it is likely that agrin signaling through β -DG which modulates AChR clustering (Kahl and Campanelli, 2003)

DG also colocalizes with major key players in synapse formation including MuSK, agrin and AChRs. MuSK, AChRs and DGs are linked together by rapsyn, which is regulated

by agrin (Moransard et al., 2003), reinforcing the importance of DG in agrin-mediated signaling and AChR stability (Apel et al., 1995).

Curiously, biglycan may provide another link between DG and agrin-MuSK signaling. Biglycan is able to bind both α -DG (Bowe et al., 2000) and MuSK, potentially regulating agrin-induced MuSK phosphorylation. Consistent with this, biglycan-null mice exhibit a decrease in MuSK expression and in the stability of AChR clusters (Amenta et al., 2012). Biglycan, however, does not appear necessary for the development of synapses and pre-patterning but is prominent at mature synapses (Amenta et al., 2012) which suggests that DG may function downstream of agrin signaling.

DG signaling is also able to influence AChR aggregation via an agrin-MuSK independent pathway (Montanaro et al., 1998). The extracellular domain of DG is capable of targeting laminin to AChRs (Tremblay, 2006). Laminin is an ECM protein capable of binding DG and stimulating AChR clustering independent of the agrin-MuSK pathway (Montanaro et al., 1998), and functions downstream of agrin and rapsyn by coordinating the assembly of NMJs (Jacobson et al., 2001).

Although DG plays a crucial role in synapse stability, there has been some debate over whether it can function as a phosphorylatable receptor. β -DG contains a PPxY site in its C-terminus, which is a conserved tryptophan (WW) binding motif (Sotgia et al., 2001). It would appear that DG expressed in muscle is not detectably phosphorylated, likely due to the fact that this site is obstructed by another protein, either dystrophin or the dystrophin

homolog utrophin, which associates with the cytoskeleton and the PPxY site (Russo et al., 2000). However, DG expression is not limited to muscle and it is possible that in other tissues it is fully capable of being phosphorylated. Experiments completed in Chinese hamster ovary (CHO) cells, wherein a full-length construct of the β-DG molecule was expressed, demonstrated that the PPxY site can be phosphotyrosylated by Src and this may act as a regulatory switch for proteins that can bind this site such as growth factor receptor-bound protein (Grb2; Sotgia et al., 2001). Grb2 is an adaptor protein important in linking the ECM to signal transduction pathways leading to cytoskeletal reorganization and has been demonstrated to co-immunoprecipitate with DG in muscle and brain (Yang et al., 1995). Previous studies have shown that in non-muscle cells tyrosine phosphorylation of β-DG recruits and directly mediates association with Grb2 (Cavaldesi et al., 1999). Grb2 functions upstream of Raf-MEK-ERK of the mitogen-activated protein kinase pathway (MAPK; (Spence et al., 2004)). DG has also been implicated in directly interacting with components of the ERK-MAPK signaling cascade, specifically mitogen-activated protein kinase kinase (MEK; (Spence et al., 2004)), as well as indirectly with the phosphatidylinisitol-3 kinase (PI3K)-Akt signaling cascade (Xiong et al., 2009), important in cytoskeleton reorganization and cell survival, respectively. These interactions with DG may also be important for pathway cross-talk via MAPK in the agrin-MuSK pathway or the neuregulin signaling pathway (see *Neuregulin* below, section 1.3) in various tissues and perhaps other signaling pathways that activate MAPK and control a range of cellular processes (Spence et al., 2004; Rimer, 2011).

1.2.5 The role of agrin is not well understood in the CNS

Agrin appears to be principally involved in synaptogenesis in the CNS, though it also has roles in plasticity, axonal growth and more interestingly, the establishment of the blood brain barrier (BBB; Reviewed in Daniels, 2012). Agrin is broadly expressed in a variety of neuronal synapses and its expression is highly concentrated at interneuronal synapses (Mann and Kröger, 1996; Hilgenberg, 2002; Kröger and Schröder, 2002; Hilgenberg et al., 2006). However, at these sites it is the transmembrane form of agrin that is predominantly expressed in the CNS (Burgess et al., 2000; Neumann et al., 2001). Unlike at the NMJ, the formation of interneuronal synapses is not solely dependent on agrin (Gingras and Ferns, 2001). Secreted agrin does, nonetheless, appear to be involved in the formation and maintenance of a subset of excitatory synapses in the mouse brain, including those that are cholinergic (Gingras and Ferns, 2001; Ksiazek et al., 2007).

MuSK, the agrin co-receptor, has been shown to be expressed at interneuronal synapses though its role in synapse formation here is unclear (Garcia-Osta et al., 2006). LRP4, another potential agrin receptor, is expressed in synaptic regions of the brain and has been shown to co-localize with post-synaptic density 95 (PSD-95; a protein functionally similar to rapsyn) but direct interaction with agrin has yet to be reported (Tian et al., 2006). One study suggests that it is agrin acting through MuSK, however, those results pointed toward a role in cortical synapse stability and maintenance (Ksiazek et al., 2007), even

though MuSK has been heretofore implicated in learning and synaptic plasticity through the activation of the cAMP response element binding (CREB) pathway (Garcia-Osta et al., 2006). Signaling through the α 3 subunit of a $\text{Na}^+ \text{K}^+$ ATPase (α 3NKA) channel, a functional neuronal receptor for agrin in the CNS, leads to the induction of CREB as well and enhances the signaling at excitatory synapses (Ji et al., 1998; Hilgenberg et al., 1999). The regulation of CREB through neural agrin modulates membrane potential and has been associated with memory formation and synaptic plasticity (Ji et al., 1998).

Endogenously expressed Tm-agrin may also play a role in influencing synapse formation in the CNS through the regulation of dendritic filopodia (McCroskery et al., 2006; 2009). Tm-agrin clustering positively regulates filopodia, which are known to promote synapse formation. The mechanism by which Tm-agrin achieves this is not well understood though it has been shown to be dependent on the activation of Rac and cdc42 via the adhesive effect mediated by the GAG chains on agrin (McCroskery et al., 2006; Lin et al., 2010). Additionally, Tm-agrin's association with lipid rafts results in the subsequent activation of signaling pathways involving proto-oncogene tyrosine-protein kinase (Fyn) and MAPK (Ramseger et al., 2009). As such, it has been proposed that Tm-agrin itself may act as a receptor or co-receptor that initiates these processes (Annies et al., 2006; Ramseger et al., 2009). Recent evidence has also demonstrated that Tm-agrin signaling through α 3NKA is responsible for the differentiation and survival of interneurons in the olfactory bulb of the brain (Burk et al., 2012). Therefore, MuSK and α 3NKA appear to function independently but

co-operatively to activate the CREB pathway and Tm-agrin can potentially function as a receptor through its GAG chains to mediate agrin's effects in the brain.

1.3 Neuregulin

The neuregulin (NRG) family of genes is composed of 4 members: *NRG1*, *NRG2*, *NRG3* and *NRG4*. However, unless otherwise specified the term “NRG” or “neuregulin” in this thesis will be in reference to *NRG1* derived proteins. The remaining three genes do not, as of this writing, play a significant role in these systems. NRGs, as they are now all called, were initially isolated from a variety of tissues by multiple labs. As a result multiple proteins from the same gene bore names reflecting differing functions. These include: heregulin (HRG; (Holmes et al., 1992) or neu differentiation factor (NDF; (Peles et al., 1992; Wen et al., 1992), acetylcholine receptor-inducing activity (ARIA; (Falls et al., 1993), glial growth factor 2 (GGF2; (Goodearl et al., 1993; Marchionni et al., 1993) and sensory and motor neuron-derived factor (SMDF; (Ho et al., 1995). These names indicate the variety of roles that NRGs has been found to be involved in which is the result of alternative splicing.

1.3.1 Alternative splicing of Neuregulin results in many isoforms

There are many NRG1 isoforms (at least 16 structurally distinct proteins), which result from multiple promoters and alternative splicing (Meyer et al., 1997). NRGs are characterized by

their EGF-like domain and N-terminal sequence. The conserved EGF-like domain (α or β) is used to activate receptors and common to all isoforms. The N-terminal sequence (Type I, II, III) is differentiated by an immunoglobulin-like domain or a cysteine-rich domain. Type I NRGs contain an extracellular, immunoglobulin-like domain. Type II NRGs contain the previously mentioned domain in addition to a kringle domain. Type III NRGs contain a cysteine-rich domain. Only NRGs containing the immunoglobulin-like domain (Type I and II) are cleaved to become secreted forms, while Type III remains a membrane-bound protein. These NRGs differ not only in structure but also in function.

1.3.2 Neuregulin is originally described as the inducer of post-synaptic proteins

Of particular interest to this thesis is ARIA, a Type I NRG and 42 kDa glycoprotein, first isolated from chick brain. Here ARIA was found to not only aggregate AChRs but also increase the expression of the α -subunit of AChRs in chick myotubes (Usdin and Fischbach, 1986; Harris et al., 1988; Falls et al., 1990; Chu et al., 1995; Jo et al., 1995). ARIA was also found to increase α -, γ - and δ -subunit AChR mRNA levels 2-fold and ϵ -subunits up to 10-fold in mouse myotubes (Martinou et al., 1991). ARIA has been demonstrated to induce the phosphorylation of a 185 kDa tyrosine kinase receptor protein (Corfas et al., 1993; Falls et al., 1993) related to epidermal growth factor receptor (EGFR; Holmes et al., 1992;

Marchionni et al., 1993). This receptor was later described as being part of the erbB family of receptors (Reviewed in Fischbach and Rosen, 1997).

1.3.3 Neuregulin signals through the erbB family of receptors

NRGs bind to their receptors via the EGF-like domain (Meyer et al., 1997) and are the functional ligands for the EGF-receptor erbB family of tyrosine kinases (Reviewed in Yarden and Sliwkowski, 2001). The erbB family is comprised of four proteins: EGFR, erbB2, erbB3 and erbB4 of which only the last three are expressed in skeletal muscle (Zhu et al., 1995). NRG binding to erbB induces the formation of a functional erbB hetero- and/or homodimers and binds with a specific affinity for erbB3 or erbB4 (Carraway and Cantley, 1994). This results in the tyrosine phosphorylation of erbB2 heterodimers or erbB4 homo- and/or heterodimers; erbB3 appears to have little tyrosine kinase activity (Zhu et al., 1995). ErbB2 and erbB3 heterodimers are the most common at NMJs but do not appear to be necessary for NMJ formation (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995; Riethmacher et al., 1997) However, it appears that erbB2 is necessary for the observed increase in the expression of synaptic protein (Burden, 1998; Sanes and Lichtman, 1999; Leu, 2003).

NRG signaling through erbB receptors induces the expression of AChR transcription by activating the Ras/MAPK and PI3K pathways and activates the N-box promoter (Duclert and Changeux, 1995; Koike et al., 1995; Tansey et al., 1996; Altiock et al., 1997; Fromm and

Burden, 1998). Both of these signal transduction pathways appear to be necessary for the induction of AChR gene expression (Tansey et al., 1996; Altiock et al., 1997). Activation of ERK (a member of the MAPK pathway) promotes the binding of the heterodimer GA-binding protein (GABP) α and β , an Ets transcription factor, to the regulatory element termed N-box (Fromm and Burden, 1998; Schaeffer, 1998; Fromm and Burden, 2001). NRG can induce transcription in the absence of phosphorylated GABP α , suggesting GABP β or yet another molecule may be involved in this process more directly (Jaworski et al., 2007; Herndon and Fromm, 2008).

The regulation of AChRs at the synapse by the N-box promoter is not unique. This binding domain is also found relative to, and as a regulatory element of, AChE, MuSK and erbB proteins (Lacazette et al., 2003). Interestingly, agrin has also been shown to activate the N-box regulatory sequence suggesting a convergence in pathways between agrin-MuSK and NRG-ErbB signaling pathways (Lacazette et al., 2003). As previously discussed, agrin signaling can also activate MAPK pathways which could lead to GABP activation. In addition MuSK expression at the synapse is regulated by the presence of an N-box in the *musk* promoter and regulated by agrin through the activation of c-Jun N-terminal kinase (JNK), which has been shown to phosphorylate GABP (Figure 5A). It has been suggested that erbB4 expressed by muscle could account for the activation of synapse specific genes, but little evidence has lead to this conclusion directly (Leu, 2003).

1.3.4 Neuregulin signaling may be dispensable for synapse formation

Although initial studies suggested NRG to be a key factor in gene expression of synapse-specific molecules, one study has shown otherwise. Yang and colleagues (2001) questioned the impact of NRG signaling on NMJ formation using HB9 mice and the cre-lox recombinase system, which selectively targets proteins in specific cell types, to generate and circumvent the lethality of complete KOs of agrin or neuregulin. In HB9 mutant mice the HB9 transcription factor has been inactivated, resulting in motor neurons that remain undifferentiated. HB9 mutant mice do not have motor neurons innervating their muscle and as such there is no source of neural signals for synaptic differentiation. A conditional KO of MuSK in HB9 mutants demonstrates that although AChR expression is still present, no clustering is detected, suggesting that neuronal signals are dispensable for early patterning of AChRs and MuSK is responsible for patterning in the absence of motor innervation (Yang et al., 2001). This study also suggests that AChR expression patterns do not change in the absence of agrin or NRG but do change for MuSK (Yang et al., 2001). It is possible that muscle agrin, shown to co-localize with MuSK and AChRs, initially clusters MuSK and then rapsyn activates MuSK, thus inducing the clustering and expression of these AChRs pre-innervation (Yang et al., 2001) (Figure 5B). Given these results, agrin, NRG and ACh signaling post-innervation would then refine and maintain aggregates, induces local sub-synaptic expression of post-synaptic molecules, and inhibit the expression of extra-synaptic AChRs, respectively (Yang et al., 2001) (Figure 5A). A role for NRG post-innervation was strengthened by the experiments of Jaworski et al., (2006). Conditional inactivation of the

two cellular sources of NRG, specifically in motor neurons and muscle, revealed a normal pattern of synaptic molecule expression (Jaworski and Burden, 2006). Thus the strongest evidence is for agrin and agrin-MuSK signaling being primarily responsible for AChR patterning and expression and not NRG though this does not leave out a supportive role for NRG.

1.3.5 Neuregulin-erbB4 has many roles in the CNS

NRG, and its receptors, are highly expressed in the brain. NRG signaling in the CNS has been implicated in many important neurodevelopmental processes e.g. cell survival and migration, interneuronal synaptic stability, myelination, maturation of dendritic spines, synaptic plasticity and the regulation of acetylcholine and various neurotransmitter receptors such as AChR, GABAR, and GluR (Huang et al., 2000; Li, 2001; Liu et al., 2001; Mei and Xiong, 2008; Williams et al., 2008; Cui et al., 2013). Curiously, erbB4 is the least described but most predominant erbB receptor enriched in the post-synaptic densities of the brain (Garcia et al., 2000; Gerecke et al., 2001). NRG binds with high affinity to erbB4 and leads to the activation of the MAPK and PI3K pathways (Huang et al., 2000; Mei and Xiong, 2008). ErbB4 homodimerization does indeed form a functional receptor capable of signaling and leads to tyrosine phosphorylation in response to NRG stimulation (Carraway and Cantley, 1994; Ma et al., 2003). This is achieved by the recruitment of erbB4, and adaptor proteins Grb2 and Shc, to neuronal lipid rafts where PSD-95 also co-localizes. Disruption of these

lipid raft signaling platforms inhibits NRG-induced ERK activation (Ma et al., 2003). The association of erbB4 with post-synaptic densities further supports its role in synaptic stability. The NRG-induced up-regulation of neurotransmitter receptor expression in the CNS is also analogous to what occurs at the NMJ. Thus far erbB4 has been demonstrated to be important in many diseases including schizophrenia, Alzheimer's, amyotrophic lateral sclerosis (Chaudhury et al., 2003; Corfas et al., 2004; Takahashi et al., 2013).

1.4 The regulation of inflammation by immune and nervous mechanisms

1.4.1 Immune responses are divided

Immune responses are categorized into innate, the first line of host defense, and adaptive (or acquired), those that include cell-mediated and humoral immunity. Innate immunity is mediated by antigen-presenting cells that recognize pathogen-associated molecular patterns (PAMPs) present on pathogens via a number of Toll-like receptors (TLR) that they express. Chemical signals secreted by antigen-presenting cells function to eliminate pathogens but also activate the differentiation of lymphocytes (T cells and B cells) signifying a transition from an innate to an adaptive immune response. Activated antigen-presenting cells undergo cytoskeletal reorganization and form immune antigen-specific synapses with naïve lymphocytes (Figure 1C) that become activated in the process (Pulecio et al., 2010). The adaptive immune response is characterized by antigen-specific lymphocytes reacting to an antigen and the development of immunological memory. These two responses are temporally

segregated though there is some overlap and antigen-presenting cells are important in the primary and secondary response (Santana and Esquivel-Guadarrama, 2006). Here I specifically focus on one of the primary and early responses of the innate immune system: inflammation.

1.4.2 Inflammation is mediated by the innate immune system

Inflammation is an immediate innate immune response designed to defend the body from pathogenic invasion. It activates antigen-presenting cells, recruits effector cells and contains infection until the initiating agent is removed. The signaling mechanisms underlying inflammatory responses are complex and involve numerous molecular players that have been extensively reviewed (please see (Beutler, 2000; Creagh and O'Neill, 2006; Foster and Medzhitov, 2009; Medzhitov, 2010; Arthur and Ley, 2013)). Due to the comprehensive literature on the subject, here I present a simplified version of the typical inflammatory response.

Bacterial products, such as lipopolysaccharide (LPS), cause local tissue macrophages to activate and drive the inflammatory response by producing an array of cytokines including tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6 as well as other mediators (Figure 5A) (Kracht and Saklatvala, 2002; Fujihara et al., 2003). LPS, through the activation of TLR4, induces inflammation primarily by activating the transcription factor nuclear factor- κ B (NF- κ B; Hayden and Ghosh, 2004). LPS, pro-inflammatory cytokines and chemokines

act locally, activating pre-existing macrophages *in situ* to produce vasodilators, vascular endothelial cells to express adhesion molecules and by recruiting circulating neutrophils and later monocytes, the precursors to macrophages, to the site of infection for pathogen elimination and subsequent antigen presentation to lymphocytes (Figure 6A).

Adhesion molecules expressed on the surface of activated endothelial cells and increased vascular permeability allow for recruited leukocytes to “roll” over the endothelium, increasing and strengthening their interactions prior to migrating across the endothelial wall via extravasation (Vestweber, 2012). Extravasation occurs in four stages: rolling adhesion, tight binding, diapedesis and migration. Enzymes, such as matrix metalloproteinases (MMPs), secreted by activated cells aid this process by loosening junctions and breaking down ECM proteins in the basement membrane (Sternlicht and Werb, 2001). Leukocytes that have migrated across the epithelium can phagocytose the infecting agent, process the antigen and express it on their cell surface. One exception to this rule is at the BBB. The CNS tightly regulates immune cell entry into the brain by the BBB, thus preventing systemic or peripheral immune cells and molecules from infiltrating the brain. The brain is in this sense an immune privileged site at which most immune responses are mediated by microglia, which are the primary immune cells and macrophages of the brain (Hickey, 2001; Nguyen et al., 2002; Streit et al., 2004; Engelhardt, 2006; Graeber et al., 2011; Muldoon et al., 2013).

Inflammation is regulated via positive and negative feedback mechanisms. Pro-inflammatory cytokines like TNF- α and IL-1 β act via a positive feedback mechanism and if the infectious agent overwhelms the resident macrophages and number of effector cells

recruited, there is an over-production of these cytokines resulting in damaging effects. Surrounding cells, such as endothelial cells, are also sensitive to these cytokines and can become activated further perpetuating these effects. TNF- α , IL-1 β and IL-6 released systemically when the infection is no longer contained results in fever and acute phase response by acting on the hypothalamus, spleen and liver. Prolonged secretion of large amounts of TNF- α can be damaging and can potentially lead to septic shock and organ failure. Activated macrophages also produce the anti-inflammatory cytokine IL-10, which is a negative feedback regulator synthesized in homeostatic control of inflammation. IL-10 acts in an auto- and paracrine manner to inhibit the synthesis of pro-inflammatory cytokines, such as TNF- α , as the infection is eliminated (Williams et al., 2004b). Quite simply then, local inflammation is a balancing act seeking to clear infection while limiting the scope of the response and the extent of collateral damage.

1.4.3 Acetylcholine regulates inflammation via the cholinergic anti-inflammatory pathway

Widespread inflammatory responses can be regulated by a neuro-immune axis termed the cholinergic or nicotinic anti-inflammatory pathway (Borovikova et al., 2000). The cholinergic anti-inflammatory pathway is a controlled mechanism that aims to keep localized inflammation from becoming systemic, thus preventing potentially lethal effects such as septic shock and organ failure (Ulloa, 2005). This bidirectional pathway is mediated by the

binding of ACh, synthesized by the efferent arm of vagus nerve, to the homopentameric $\alpha 7$ nAChR present on macrophages and monocytes (Wang et al., 2003a).

The activation of the $\alpha 7$ nAChR by ACh initiates a signaling cascade that prevents the nuclear translocation of NF- κ B and activation of the Jak2-STAT3 pathway (see figure 6B). This inhibits the production of pro-inflammatory cytokines such as TNF- α and IL-1 β (Yoshikawa et al., 2006), but does not affect production of the anti-inflammatory cytokine IL-10 (Ulloa, 2005; Gallowitsch-Puerta and Pavlov, 2007).

ACh also appears to control inflammation by affecting other effector cells involved in this innate immune response. Vascular endothelial cells also express the $\alpha 7$ nAChRs and activation of this receptor in the presence of LPS inhibits the expression of cell adhesion molecules and the production of chemokines, which influence the recruitment and migration of leukocytes during inflammatory responses (Saeed et al., 2005). Collectively, it appears that ACh can mediate the inhibition of macrophage, monocyte, neutrophil and endothelial cell activation by LPS through the activation of the $\alpha 7$ nAChRs. Due to the wide expression of AChRs in non-neuronal, non-excitatory cells (Wessler et al., 1998), where the ACh molecule acts more like a cytokine (autocrine/paracrine) than a neurotransmitter (paracrine) (Matthay and Ware, 2004), current research aims are to exploit the nicotinic anti-inflammatory pathway to treat autoimmune disorders (Pavlov and Tracey, 2006). Other inflammatory conditions such as periodontitis, psoriasis, sarcoidosis, ulcerative colitis, Crohn's disease, and septic shock can also be targeted (Scott and Martin, 2006). However, there are cytotoxic

effects of therapeutically using nicotine, a high affinity agonist of the α 7nAChR, as treatment for such disorders (Saeed et al., 2005). As such, there is an obvious need to investigate possible alternatives that can regulate inflammatory signaling pathways, perhaps upstream of α 7nAChR activation. Determining how agrin and NRG are involved in regulating inflammation may provide for this alternative treatment.

1.5 Agrin and NRG in the immune system

Recent evidence suggests, roles for agrin and NRG in immune system function. Agrin and α -DG (Mazzon et al., 2012), and α 7nAChRs (Serobyan et al., 2007), have been individually described to be important in development of the hematopoietic system. Agrin and α -DG also appear essential to the survival of hematopoietic stem/progenitor cells (HSPC) that give rise to all hematopoietic cells which include monocytes, macrophages, neutrophils, T and B cells (Mazzon et al., 2012). Finally, agrin has been shown to regulate some immune cell functions on T lymphocytes (Khan et al., 2001; Jury et al., 2007; Kabouridis et al., 2012) and most recently on monocytes (Mazzon et al., 2012). These results identify agrin as an important molecule in development beyond the nervous system.

In T lymphocytes, agrin has been implicated in regulating the threshold for lymphocyte activation by acting as a co-stimulatory molecule and reorganizing lipid rafts (Khan et al., 2001). Agrin appears to act in an autocrine/paracrine fashion in these cells (Khan et al., 2001), unlike at the NMJ and similar to ACh in non-neuronal tissues. Also, in

lymphocytes it appears that agrin's clustering capability is not determined by an eight amino acid insert at the Z site, but rather by a deglycosylation event resulting from cellular activation. Remarkably, this modification yields an active agrin molecule capable of clustering AChRs in muscle (Khan et al., 2001). This result also suggests that other agrin molecules, expressed in non-neuronal tissues, can induce cytoskeletal re-organization. Agrin signaling has been found to activate the MAPK pathway in T lymphocytes and augment IL-10 production, though curiously it does not activate JNK or NF- κ B (Jury et al., 2007). In monocytes, agrin has been demonstrated to be necessary for cell survival and function, which is likely mediated by signaling through α -DG (Mazzon et al., 2012). Interestingly, agrin expressed on antigen-presenting cells, has also recently been implicated in multihistocompatibility complex (MHC) clustering and antigen presentation, as well as augmenting the production of pro-inflammatory mediators (Kabouridis et al., 2012). This suggests an important regulatory role for agrin in immune cells.

NRG/erbB4 signaling has been demonstrated to be involved in a variety of cellular and immune functions. Since NRG is highly expressed in glial cells, such as microglia, it is logical to investigate its roles in CNS immune regulation. Microglia are the macrophages of the CNS and are activated in response to physical and bacterial insult resulting in neuroinflammation, which is characterized by the synthesis of a variety of pro-inflammatory mediators. NRG has been reported to protect microglial cells against inflammation-induced cell death (Dimayuga et al., 2003) and appears to antagonize apoptosis by activating the PI3K pathway (Cui et al., 2013). Recently, we have also implicated NRG in a neuroprotective role

on microglia. NRG induced upregulation of $\alpha 7$ nAChR on LPS-challenged microglia and lead to a significant decrease in pro-inflammatory mediators (Mencel et al., 2013). Not only does this suggest that NRG may augment the cholinergic anti-inflammatory pathway in microglia but this further supports claims of NRG's involvement in neuroprotection in the CNS.

1.6 Rationale and Objectives

Although $\alpha 7$ nAChRs have been implicated in the cholinergic anti-inflammatory pathway, and agrin and NRG have been described to be important for cell survival in the peripheral immune system and the brain respectively, the connection between agrin, NRG and AChRs has not been investigated. Using what we know about these molecules at nervous system synapses may provide insights into their role in the immune system. I have investigated roles for agrin and NRG in macrophages and microglia, respectively, and in inflammation as a whole. Macrophages are one of the primary responders to infection and are essential to inflammatory processes in both the periphery and the brain. Inflammation is, on some level, at the forefront of an incredible number of chronic conditions and diseases. Since AChRs are ubiquitously expressed on all immune cells and mediate a cholinergic anti-inflammatory response initiated by ACh as part of a neuro-immune axis, neuron-derived molecules regulating immune responses are increasingly important. The major question is: Do these neurotrophic molecules play a similar role on macrophages as they do on muscle cells? Are they and their receptors expressed endogenously? And does this role affect inflammation as a

result of the augmentation of AChRs? Here I support the recently proposed notion that NRG and agrin are novel players in innate immunity. Treating immortalized microglial and macrophage cell lines with these neurotrophic molecules to determine their effect on the expression of AChRs as well as pro-inflammatory and anti-inflammatory cytokines has shown that both NRG and agrin can augment AChR and cytokine levels, in microglia and macrophages respectively. This gives insight into their possible role in innate immune response.

Although it appears there is a difference in agrin and NRG function and a compensatory mechanism is prevalent, in the peripheral versus neuronal immune systems, respectively, both agrin and NRG are here implicated in mediating an anti-inflammatory response in concert with the cholinergic anti-inflammatory pathway. Here I will show that agrin treated macrophages express increased levels of endogenous agrin and α 7nAChRs and cluster together on the cell surface, and that DG, α 7nAChRs and endogenous agrin interact in macrophages. I will also show that agrin treated macrophages and neuregulin treated microglia, that have been challenged with LPS, exhibit decreased levels of pro-inflammatory cytokines and agrin can increase anti-inflammatory IL-10 in macrophages. In summary, agrin and NRG reduce mediators of inflammation, in concert with the cholinergic anti-inflammatory pathway, in macrophages and microglia *in vitro*.

Chapter 2

Materials and Methods

2.1 Cell Lines and Culture

The RAW264.7 murine macrophage cell line (TIB-71; ATCC) was cultured in RPMI 1640 (Wisent, St. Bruno, QC) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Wisent), 100IU/ml penicillin (Wisent) and 100mg/ml streptomycin (Wisent). The BV-2 murine microglial cell line (received as a gift from Dr. Michael J. Strong, University of Western Ontario, London, ON) was cultured in the same manner as the RAW264.7 cell line. The C2C12 murine myoblast cell line (CRL-1772; ATCC) was cultured in low glucose Dulbecco's modified Eagle's medium (DMEM; Wisent) supplemented with 10% FBS, 100IU/ml penicillin and 100mg/ml streptomycin. When cells reached 80% confluency, culture media was changed to DMEM supplemented with 2% horse serum (HS; Wisent), 100IU/ml penicillin and 100mg/ml streptomycin to allow for differentiation of myoblasts to myotubes. All cell lines were grown at 37 degrees Celsius and 5% CO₂ with the exception of C2C12 cells which were grown at 8% CO₂.

E7 hybridoma cells were purchased from the Developmental Studies Hybridoma Bank (DSHB; University of Iowa, Iowa City, IA) for β-tubulin antibody production. E7 cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS and 0.05mg/ml gentamycin. Cells remained in culture 10-14 days for efficient and maximum Ig secretion,

then the cells were centrifuged and discarded. Supernatant was retained, filter-sterilized and stored at 4 degrees Celsius until needed.

2.2 Cell Culture Treatments

Cells were cultured as described above in section 2.1, until approximately 80-90% confluence before treatments, and cell growth media was refreshed prior to treatments. Macrophages and microglia were either pre-treated for 5 or 30 minutes, respectively, with 1mM pyridostigmine bromide (Sigma-Aldrich, St. Louis, MO), an acetylcholinesterase inhibitor, and 100 μ M acetylcholine chloride (ACh; Sigma-Aldrich) or left untreated [adapted from ((Borovikova et al., 2000; Wang et al., 2003a; Shytle et al., 2004)) to remain consistent with previous studies]. Then all macrophages were treated for four hours with 500pM agrin (rrC-Ag 3,4,8, R&D Systems, Minneapolis, MN), 100ng/ml lipopolysaccharide (LPS; *Escherichia coli* 0111:B4, Sigma-Aldrich, St. Louis, MO), both agrin and LPS, or left untreated, unless otherwise stated. All microglia were treated for four hours with either 1nM neuregulin (NRG; rh-NRG-1, Shenandoah Biotechnology, Warwick, PA), 100ng/ml LPS, both NRG and LPS, or left untreated, unless otherwise stated.

2.3 Protein Extraction

Following treatment of macrophages, cells were briefly washed twice with cold Dulbecco's phosphate-buffered saline (D-PBS; Wisent), and harvested in D-PBS with a rubber

policeman. Cells were then collected by centrifugation at 1500 rpm for 10 minutes at 4 degrees Celsius. Supernatants were discarded and cell pellets were re-suspended and lysed in protein extraction buffer [25mM Tris-HCl pH 7.5, 25mM Glycine, 150mM NaCl, 1X Complete Protease Inhibitor Cocktail (Roche, Mississauga, ON), 1% Triton X-100 (Sigma-Aldrich) and 5mM EDTA pH 8.0 (Quality Biological Inc., Gaithersburg, MD)] on ice for 15 minutes. The supernatant was collected and centrifuged at 14000 rpm for 5 minutes at 4 degrees Celsius.

2.4 SDS-PAGE and Immunoblot Analyses

Protein extracts were quantified using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Mississauga, ON) for equal protein sample loading. SDS-PAGE and immunoblots were performed according to methods previously described (Jacobson et al., 1998). Protein samples were electrophoretically separated on 6, 8 or 10% SDS-PAGE, and subsequently transferred to a nitrocellulose membrane. Membranes were subjected to Ponceau S (Sigma-Aldrich) stain to confirm efficient transfer of proteins then blocked overnight with either 5% non-fat dehydrated milk (NFDM) (for dystroglycan, agrin, and β -tubulin), 5% bovine serum albumin (BSA; Wisent) (for STAT3), or 2.5% BSA/2.5% NFDM (for α 7nAChRs) in TBS-T [10mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20]. Membranes were then incubated in primary monoclonal antibodies against agrin (1:1000, m247, m33; Cedarlane), α -DG (1:1000, 11H6; Millipore), β -DG (1:1000, NCL-b-DG; Leica Biosystems Newcastle, Ltd., Newcastle, UK), STAT3 (1:1000, Cell Signaling Technology, Inc., Danvers, MA),

polyclonal antibody against α 7nAChR (1:1000, ab23832; Abcam, Cambridge, MA), and an e7 hybridoma-conditioned medium for loading control (1:250, β -tubulin; DSHB).

Horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1000-1:2000; Amersham, Arlington Heights, IL) were used to visualize protein-specific binding. Immunoreactivity was detected using ECL (Pierce, Rockford, IL) and captured on autoradiography film (LabScientific, Highlands, NJ). Molecular weight markers were used to estimate protein size (Fermentas, Burlington, ON). C2C12 protein extracts were used as a positive control for agrin and dystroglycan. For semi-quantitative analysis, the relative intensity of protein bands was determined by densitometry using NIH ImageJ software (NIH).

2.5 Blocking experiments

α -DG was blocked with the monoclonal antibody 11H6 (sc-53987; Santa Cruz Biotechnology, Santa Cruz, CA). This antibody was diluted 1:100 in cell culture medium one hour prior to treatments (adapted from (Jacobson et al., 1998)).

2.6 Flourescent staining of α 7nAChRs and co-staining of agrin for immunocytochemistry

Cells were grown on etched glass coverslips overnight. Cells were treated as indicated in section 2.2. After treatment, 0.2 μ g/ml α -bungarotoxin-tetrarhodamine (α -BTX-Rho; T1175;

Life Technologies Inc., Burlington, ON) was added to cultures at room temperature for one hour to stain for α 7nAChRs. Cells were washed twice with cold D-PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich) prior to mounting onto glass slides Slowfade Gold mounting media (P36934; Life Technologies, Inc.).

For co-staining of α 7nAChRs and agrin, cells were first stained for α 7nAChRs as indicated previously, washed and fixed with 2% paraformaldehyde (Sigma-Aldrich) to adjust for the permeabilization step. Cells were permeabilized with 0.3% Triton X-100/PBS and subsequently blocked with 10% HS/PBS. Cells were stained for agrin with a monoclonal antibody (1:200, m33; Cedarlane) diluted in 5%HS/PBS, overnight at 4 degrees Celsius, were washed in D-PBS, and then probed with a secondary antibody conjugated to FITC (1:200; Jackson Immunoresearch Laboratories, Inc., Westgrove, PA). Cells were then mounted onto glass slides with Slowfade Gold mounting media (Life Technologies, Inc.) prior to viewing.

Slides were viewed and imaged using Zeiss Axiovert 200 microscope (Zeiss, Mississauga, ON), Qimaging retiga 1494 digital camera (Qimaging, Burnaby, BC), and Openlab software (Improvision, Waltham, MA), or Nikon Eclipse 90i fitted with a Nikon D-eclipse C1 scan head using Nikon EZ-C1 software (Nikon Canada Inc., Mississauga, ON) for confocal microscopy, at a final magnification of 400x. Co-localization clusters were counted from a merged image of co-stained cells from at least 5 random fields using NIH ImageJ software (NIH, Bethesda, MD). Values are expressed per cell to take into account any difference in cell density between cultures. Clusters were counted independent of size.

2.7 Immunoprecipitation (IP) of α 7nAChRs and co-IP of proteins

α 7nAChRs were immunoprecipitated from protein samples by incubation with 1 μ g biotin-XX- α -bungarotoxin (Life Technologies) at 4 degrees Celsius for one hour with gentle rocking. Upon the addition of 50 μ l streptavidin-sepharose beads (Upstate Biotechnology, Lake Placid, NY), samples were incubated an additional hour at 4 degrees Celsius with gentle rocking.

Similarly, agrin and dystroglycan were immunoprecipitated from protein samples using a monoclonal anti-agrin antibody (1 μ g, m247 or m33; Cedarlane) and monoclonal 11H6 anti-dystroglycan antibody (Santa Cruz), respectively, and 50 μ l recombinant protein-G agarose beads (Invitrogen).

Beads were then washed with a high-salt IP wash buffer [50mM NaCl, Tris-HCl pH 8.0, 0.5% Triton X-100] and reserved for western blot analysis.

2.8 Analysis of secreted TNF- α , IL-6 and IL-10 by ELISA

The concentration of secreted TNF- α , IL-6 and IL-10 in cell culture media was determined by ELISA, following the manufacturer's protocol (88-7324, 88-7062, 88-7105; eBioscience, San Diego, CA). Cell culture media harvested from treated and untreated macrophages and microglial cells was stored at -80 degrees Celsius until needed. Cells were treated as previously indicated (see section 2.2).

2.9 Statistical Analyses

All experiments were completed in triplicate or more and measurements are expressed as means \pm SE, unless otherwise indicated. Statistical analysis was performed using Student's *t*-test to compare two groups. Statistical significance of differences between groups was analyzed by ANOVA with Tukey's Post-Hoc test for multiple comparisons, unless otherwise indicated. Differences were considered significant when *P* values were less than 0.05, using Kaleidagraph 4.1.1 (Synergy Software, Reading, PA).

Chapter 3

Results

3.1 Agrin

3.1.1 Agrin is expressed on macrophages and appears to be self-regulated

Agrin expression has been previously described in a variety of neuronal and non-neuronal cells (Smith et al., 1987; Magill-Solc and McMahan, 1990) where it appears to mediate a significant number of cellular responses. In T cells, cell activation induces a deglycosylation event which increases the ability of endogenous agrin to aggregate receptors (Khan et al., 2001). My initial investigations centered on whether agrin is endogenously expressed by macrophages and if it mediates the activation of macrophages as it does in T cells. To do this a functional macrophage cell line sensitive to LPS was utilized, RAW264.7, purchased from ATCC (Raschke et al., 1978). Initial, immunoblotting experiments with RAW264.7 extracts, indicated that agrin is endogenously expressed by the RAW264.7 murine macrophage cell line and that these cells are sensitive to exogenous agrin. The agrin in the RAW264.7 cell line is similarly expressed as that seen in the C2C12 murine myotube cell line (Figure 7). Whole cell extracts were prepared from the macrophage cell line RAW264.7 that were treated with 500pM agrin, 100ng/ml LPS or left untreated for four hours. Since agrin is secreted by all cholinergic neurons and to simulate this situation in vitro, cells were treated with a C-95 fragment agrin, described to be sufficient in mediating agrin's activity

(Gesemann, 1995; Campagna and Fallon, 2006). Cells were also treated with LPS because it is a well-described and potent activator of macrophages and for the comparison of an activated phenotype (Beutler, 2000; Fujihara et al., 2003). A four hour incubation time was chosen based on previously described agrin activity on cultured muscle (Wallace, 1988) and because four hours is within a typical time frame for an innate immune response (Medzhitov, 2007). Following electrophoretic separation and transfer to nitrocellulose, membranes were immunoblotted with a monoclonal antibody against agrin, m247, which binds close to the C-terminal splice sites on agrin (Hoch et al., 1994). The core agrin protein has a molecular weight of approximately 200kDa. In untreated, agrin and LPS-treated RAW264.7 cells, agrin is differentially expressed as shown in a representative immunoblot (Figure 8A). The agrin endogenously expressed in macrophages is likely the transmembrane form, though the expression of a secreted form cannot be excluded (Khan et al., 2001). When compared to control (untreated) RAW264.7 cells, densitometric analyses conducted on unsaturated exposures from multiple immunoblots confirmed that agrin treatments induced approximately a 2.18 ± 0.47 -fold increase in endogenous agrin expression ($n=5$, Student's *t*-test $p<0.001$; Figure 8B). This increase is similar to what is observed in muscle in response to neural agrin (Lieth and Fallon, 1993). LPS addition did not appear to significantly induce an increase in agrin expression on macrophages (1.28 ± 0.63 -fold, $n=5$; Figure 8B). However, this result provides a possible baseline for agrin induction due to classical macrophage activation by LPS. The results documented here are in agreement to what occurs in muscle and suggest this non-neural agrin isoform is regulated in a manner similar to neural agrin

(Lieth and Fallon, 1993). These results also suggest that agrin activates macrophages, though it appears this response is separate from that seen upon LPS stimulation. The agrin-induced activation and induction may be autocrine in nature and self-regulatory. Unlike in T cells, no deglycosylation event, demonstrated by a differential immunoreactivity between m33 and m247 agrin antibodies on resting and activated cells (Khan et al., 2001), was observed in macrophages (unpublished observations).

To confirm the optimal concentration of agrin for treatments at four hours I tested a variety of doses on RAW264.7 cells as illustrated by the representative immunoblot shown in figure 9A. Using densitometric analysis, macrophages treated with 500pM agrin demonstrated the largest increase in endogenous agrin expression at 1.91 ± 0.65 fold ($n=3$, $p<0.05$ ANOVA/ Fisher's LSD $p<0.05$; Figure 9B) compared to control, an increase consistent with earlier experiments. Increases beyond this concentration were not as effective with 1000pM agrin yielding only a 1.78 ± 0.13 fold increase ($n=3$, $p<0.05$ ANOVA/ Fisher's LSD $p<0.05$) and 750pM agrin yielding a 1.73 ± 0.50 fold in endogenous agrin expression.

3.1.2 Agrin mediates its effects through DG in macrophages

Agrin has been shown to bind DG in a variety of tissues (Gesemann et al., 1996; 1998). Initial experiments examined β -DG expression and demonstrated that β -DG is expressed in RAW264.7 cells, similar to that seen in C2C12 cells (Figure 10). The expression of β -DG indicates that α -DG must also be expressed as both α - and β -DG results from the post-

translational cleavage of the same protein (Ibraghimov-Beskrovnyaya et al., 1992). To determine if agrin mediates its inductive effects via DG an anti- α -DG monoclonal antibody was utilized to execute a α -DG blocking experiment (Gee et al., 1994; Jacobson et al., 1998). RAW264.7 cultures were subjected to treatment with a 1:100 dilution of the α -DG antibody 11H6 for one hour prior to the addition of agrin, LPS, both agrin and LPS, or left untreated, for four hours. A representative immunoblot demonstrates that both agrin- or LPS-induced increases in the expression of endogenous agrin are significantly reduced by blocking α -DG (Figure 11A). Densitometric analysis indicated that blocking with 11H6 reduced the expression of endogenous agrin by 83% in agrin treated cells ($n=3$, $p<0.0001$ ANOVA/Tukey HSD $p<0.001$) when compared to agrin treated alone (3.27 ± 1.29 fold, $n=3$; Figure 11B). Interestingly, blocking with 11H6 also reduced the expression of endogenous agrin by 98.5% in cultures subsequently treated with LPS for 4 hours ($n=3$, $p<0.0001$ ANOVA/Fisher's LSD $p<0.05$) when compared to LPS treated alone (1.12 ± 0.31 fold, $n=3$; Figure 4B). Taken together, these results suggest that agrin induction of endogenous agrin functions by binding to DG and this mediates agrin's downstream effects.

3.1.3 Agrin addition induces an increase in α 7nAChR expression in macrophages

At the NMJ, agrin signaling contributes to the increased expression of post-synaptic molecules, including AChRs (Jones et al., 1996; Yang et al., 2001; Lacazette et al., 2003).

α 7nAChRs are expressed on macrophages and regulate inflammation (Wang et al., 2003a).

To determine if agrin had similar inductive effects on macrophages I investigated the function of agrin on α 7nAChR expression in RAW264.7 cells. Macrophages were either treated with agrin, LPS, or left untreated, for four hours. Preliminary experiments using crude protein lysates from RAW264.7 cells indicated a low level of expression of α 7nAChR. To compensate for this α 7nAChR was precipitated with α -BTX conjugated to biotin and streptavidin-agarose beads, and subsequently probed with α 7nAChR anti-sera. A representative immunoblot illustrates that both agrin and LPS treatments induced an increase in α 7nAChR expression (Figure 12A). Densitometric analysis revealed that agrin addition induced a 3.52 ± 1.25 fold increase in α 7nAChR expression in macrophages ($n=3, p<0.05$, Student's *t*-test) while LPS only induced a non-significant increase of 1.40 ± 0.38 fold (Figure 12B). This data suggests that agrin induces α 7nAChR expression and may function upstream of the cholinergic anti-inflammatory pathway. Whether LPS can induce an increase in protein expression of α 7nAChRs has not been previously reported in macrophages in the periphery though experiments examining LPS challenged macrophages of the brain and α 7nAChR expression remain inconclusive (Tyagi et al., 2010; Mencel et al., 2013). However, a LPS induced increase in α 7nAChR mRNA has been reported in U937 macrophages (Chernyavsky et al., 2010). Congruent with activation and taken with these observations, our results further implicate agrin in the activation of macrophages.

3.1.4 ACh addition antagonizes agrin's effects

Nerve-secreted ACh activates a Cdk5 mechanism and reportedly antagonizes agrin's effects, which results in the destabilization of AChR clusters at the NMJ (Lin et al., 2005; Misgeld et al., 2005). Since ACh has been implicated in mediating the cholinergic anti-inflammatory pathway in macrophages we then investigated if ACh would antagonize agrin's effects in macrophages. To determine the effects of ACh, RAW264.7 cells were pre-treated with 100 μ M ACh in the presence of an AChE inhibitor for five minutes, prior to treatment with agrin, LPS, both agrin and LPS, or left untreated, for four hours. A representative immunoblot shows that ACh antagonizes the effects of both agrin and LPS on RAW264.7 cells (Figure 13A). Densitometric analysis revealed that ACh pre-treated macrophages subjected to agrin addition reduced the expression of endogenous agrin by 94% when compared to agrin treated cells alone (2.13 ± 0.11 -fold, n=4, $p<0.001$ ANOVA/ Tukey's HSD $p<0.001$; Figure 13B). Curiously, ACh also reduced the expression of endogenous agrin in LPS challenged macrophages by 67% when compared to LPS alone (1.31 ± 0.25 fold, n=4, $p<0.001$ ANOVA/ Fisher's LSD $p<0.05$; Figure 13B). Experiments completed in muscle have described the antagonistic effects of ACh on agrin-induced clusters however there has been not report on the modulation of post-synaptic proteins. These results suggest an inhibitory or antagonistic role for ACh in macrophages with respect to agrin, in general.

3.1.5 Agrin addition clusters α 7nAChRs on macrophages

Nerve-derived agrin induces the aggregation of AChRs in the PNS and CNS (Reist et al., 1992; Bowe et al., 1994). α 7nAChRs have been reported to form clusters in macrophages (Wang et al., 2003a). Since our earlier results indicate that agrin may activate macrophages, similar to LPS, I next investigated if agrin induced the clustering of α 7nAChRs in macrophages. To determine if agrin has a similar effect on macrophages RAW264.7 cultures were treated using 1nM agrin for maximum effect for four hours, or were left untreated, prior to staining with a rhodamine-conjugated α -BTX probe to allow for the visualization of α 7nAChRs. Macrophages were then fixed and mounted under glass slides and subsequently visualized using fluorescent microscopy. A representative image qualitatively demonstrating an increase in the size and number of α 7nAChR clusters observed as discrete puncta on agrin treated macrophages, compared to control, is shown in Figure 14A as indicated. α 7nAChR clusters were counted per cell, independent of size, and are represented in figure 14B. Agrin induced a 2.74 ± 0.30 -fold increase in α 7nAChR clusters ($p < 0.0001$, Student's *t*-test) in macrophage relative to the spontaneous clusters observed on untreated or control cells (Figure 14B).

Spontaneous clusters observed in a small subset of untreated cells, especially at areas of cell-cell contact, were generally smaller than those observed in agrin-treated cells ($< 1\mu\text{m}$ in diameter). Agrin-induced clusters, however, are not much larger ($\sim 1\text{-}2\mu\text{m}$ in diameter) and thus would be considered microaggregates in muscle where agrin-induced aggregates are generally larger ($> 1\mu\text{m}$ in diameter and can be larger than $10\mu\text{m}$; (Wallace, 1988). Overall,

these results indicate a role for agrin-induced clustering of surface α 7nAChRs, and thus activation, in macrophages, and these results are in line with our previous results.

3.1.6 Agrin expressed on macrophages co-localizes with α 7nAChRs

Nerve-derived agrin has been shown induce the clustering of various post-synaptic molecules including endogenous agrin (Lieth et al., 1992; Lieth and Fallon, 1993), and agrin receptors such as α -DG (Sugiyama et al., 1994), and AChRs have been shown to co-localize with both (Fallon and Gelfman, 1989; Gee et al., 1994; Sugiyama et al., 1994). The interaction and clustering of receptors is important in the initiation of a variety of signaling pathways. To determine if α 7nAChRs also co-localize with agrin expressed on macrophages, RAW264.7 cultures that were treated with 500pM agrin for four hours, or left untreated, were co-stained with rhodamine-conjugated α -BTX (red) for α 7nAChRs and a primary monoclonal antibody against agrin followed by a FITC-conjugated secondary (green), respectively. Cells were visualized using confocal microscopy for improved resolution and representative images are shown in figure 15A. An artificial merge was used to visualize areas of co-localization between agrin and α 7nAChRs (shown in yellow; Figure 15A). Our results again show an increase in the size and number of agrin-induced α 7nAChRs clusters after four hours, compared to control, but also show that endogenous agrin expressed by macrophages also clusters on these in response to agrin in a similar manner. This observation is similar to what has been described at the NMJ, where muscle agrin clusters with 90% of AChRs (Lieth and

Fallon, 1993). Furthermore, agrin and α 7nAChR co-localize in both treated and untreated macrophages (Figure 15A). The puncta observed in treated macrophages are more discrete and pronounced than in untreated cells (Figure 15A). Our results also indicate that there is a 2.31 ± 0.24 -fold increase (Figure 15B, $n=3$, $p<0.0001$, Student's *t*-test) in these surface clusters of α 7nAChRs and agrin in agrin-treated macrophages. The existing puncta and few spontaneous clusters observed in untreated macrophages could be the result of endogenous agrin or pre-patterning events in these cells, similar to what occurs in muscle, as muscle agrin has low AChR clustering ability on its own (Godfrey, 1991; Lieth et al., 1992).

To confirm that the co-localization observed with confocal microscopy was a result of a direct or indirect interaction between these molecules I used co-immunoprecipitation techniques to pull down endogenous agrin, α 7nAChRs, and in addition agrin's receptor α -DG, from total protein lysates obtained from macrophages, prior to probing with agarose beads and separating the elutions via electrophoresis. Endogenous agrin interacting with α 7nAChRs and α -DG on macrophages would suggest the involvement of agrin in a novel signaling complex mediating immune responses in these cells. Figure 16 demonstrates representative blots of experiments repeated three times. Total protein extracted from cells treated for four hours, or left untreated, were subjected to immunoprecipitation with either biotin-conjugated α -BTX, anti- α -DG or anti-agrin antibodies. These lysates were then pulled down with streptavidin-agarose or protein G beads prior to being electrophoretically separated. After transfer to nitrocellulose, membranes were immunoblotted with antibodies against agrin, α -DG, and α 7nAChRs. Our results confirm a direct interaction between all of

these molecules as demonstrated in Figure 16. Interestingly, our results suggest that the relative interaction between endogenous agrin and α 7nAChRs in macrophages do not appear to differ amongst activated and unactivated macrophages, and this could suggest that macrophage activation in response to agrin does not directly initiate inflammatory pathways. We do see however, that there is an increased association of DG with agrin in immunoprecipitations completed with the m33 anti-agrin antibody, which is not seen with the m247 anti-agrin antibody, in extracts from agrin treated macrophages. The m33 antibody binds all isoforms of agrin and thus may associate better with α -DG.

3.1.7 Agrin addition does not induce the synthesis of pro-inflammatory cytokines TNF- α and IL-6 in macrophages

Since α 7nAChRs have been previously implicated in regulating inflammation via the cholinergic anti-inflammatory pathway (Borovikova et al., 2000; Wang et al., 2003a) and agrin has been shown to interact with and induce the expression of α 7nAChRs (see above), I next investigated the impact of agrin on pro-inflammatory cytokine synthesis in macrophages. To determine if agrin affected the synthesis of TNF- α , one of the key local mediators of inflammation, in RAW264.7 cells, I used a commercially available ELISA kit for TNF- α . Culture media was harvested from RAW264.7 cells that were treated for four hours with agrin, LPS, both agrin and LPS, or left untreated, in the presence or absence of ACh. Figure 17 shows that a four hour treatment with agrin alone did not result in a significant increase in the concentration of TNF- α (14.82 ± 0.70 pg/ml) when compared to

LPS alone (401.82 ± 7.39 pg/ml, n=7, $p < 0.0001$ ANOVA/Tukey HSD $p < 0.0001$) relative to untreated controls (3.73 ± 0.15 pg/ml, n=7, $p < 0.0001$ ANOVA/Tukey HSD $p < 0.0001$). Agrin induced TNF- α levels were, in fact, comparable to those seen when macrophages were treated with ACh alone (13.30 ± 2.86 pg/ml, n=7; Figure 17). Agrin treated macrophages that have also been challenged with LPS (430.55 ± 4.93 pg/ml, n=7) exhibit TNF- α levels comparable to LPS alone, even in the presence of ACh (374.16 ± 9.92 pg/ml, n=7; Figure 17). These results taken together suggest agrin has no significant impact on the synthesis of the pro-inflammatory cytokine TNF- α in macrophages.

I then investigated the effect of agrin on IL-6 levels in macrophages, as IL-6 is another common pro-inflammatory mediator that functions locally but also has more systemic effects during inflammation. Using media collected from RAW264.7 cultures that had been treated for four hours with agrin, LPS, both agrin and LPS, or left untreated as a control, I analyzed the levels of secreted IL-6 using a commercially available ELISA kit. Figure 18 illustrates that agrin does not induce the synthesis of IL-6 in the absence (0 pg/ml, n=3) or the presence of ACh (0 pg/ml, n=3), compared to control (0 pg/ml, n=3). However, agrin added to LPS challenged macrophages induced a 56.8% decrease of IL-6 levels in the absence of ACh compared to LPS challenged alone (129.44 ± 1.29 pg/ml, n=3, $p < 0.0001$ ANOVA/ Fisher's LSD $p < 0.05$; Figure 18), and a 62.5% decrease of IL-6 levels in the presence of ACh compared to LPS challenged macrophages in the presence of ACh (242.87 ± 5.93 pg/ml, n=3, $p < 0.0001$ ANOVA/ Fisher's LSD $p < 0.05$; Figure 18). Recently, agrin has been implicated in the function and survival of monocytes (Mazzon et al., 2012). In

light of this recent study, the results presented suggest a primarily anti-inflammatory role for agrin.

3.1.8 Agrin addition induces an increase in anti-inflammatory IL-10 synthesis in macrophages

Over-expression of agrin in T cells of Lupus patients has been described to promote IL-10 synthesis (Jury et al., 2007). IL-10 is a major regulator of anti-inflammatory gene synthesis.

In light of our previous results and these recent findings I sought to determine if agrin affected the synthesis of IL-10 in macrophages. ACh functioning via α 7nAChRs and cholinergic anti-inflammatory pathway has been described to not affect IL-10 synthesis (Borovikova et al., 2000). Using a commercially available ELISA kit for IL-10, I analyzed IL-10 levels in culture media harvested from RAW264.7 cells treated for four hours with agrin, LPS, both agrin and LPS, or untreated controls, in the absence and presence of ACh. Here I show that agrin alone induces a 15-fold increase in the concentration of IL-10 (142.67 ± 78.48 pg/ml, n=4) compared to control (9.44 ± 2.74 pg/ml, n=4, $p < 0.05$ ANOVA/ Fisher's LSD $p < 0.05$; Figure 19). Interestingly, ACh appears to antagonize agrin's effects as IL-10 levels are reduced by 92% in agrin treated macrophages in the presence of ACh (11.61 ± 9.05 pg/ml, n=4, $p < 0.05$ ANOVA/ Fisher's LSD $p < 0.05$; Figure 19) when compared to agrin treated alone. LPS has been previously reported to induce IL-10 synthesis in macrophages as part of a negative feedback loop and our results agree with this observation (199.48 ± 41.46 pg/ml, n=4, $p < 0.05$ ANOVA/ Fisher's LSD $p < 0.001$; Figure 19). Our results

suggest that agrin may function upstream of IL-10 signaling pathways, directly and/or indirectly supporting the cholinergic anti-inflammatory pathway, by initiating survival pathways upon cell activation.

3.1.9 Agrin addition induces an increase in the anti-inflammatory regulator STAT3

Next I investigated how agrin might mediate its anti-inflammatory functions. One mechanism in which ACh acting through the cholinergic anti-inflammatory pathway mediates its anti-inflammatory functions is through the Jak2-STAT3 pathway (Marrero and Bencherif, 2009). STAT3 is a regulator of anti-inflammatory action and also responsible for activating the synthesis of IL-10 through a positive feedback loop and inhibition of LPS-induced TNF- α and IL-6 synthesis by mediating the function of IL-10 (Williams et al., 2004a). DG signaling has been reported to modulate STAT5 in some tissues (Leonoudakis et al., 2010). For these reasons it was very attractive to investigate agrin's effect on STAT3. To determine if agrin regulates STAT3 activation in macrophages, RAW264.7 cells were either treated with agrin, LPS, both agrin and LPS, or left untreated, for 4 hours, prior to protein extraction. Upon electrophoretic separation of total protein lysates and immunoblotting with a monoclonal antibody against STAT3 I determined that agrin had no effect on STAT3 protein levels at this time point (data not shown). However, macrophages treated with agrin, LPS, both agrin and LPS, or left untreated, for 15 minutes, did exhibit differential expression

of STAT3 as demonstrated by the representative immunoblot shown in Figure 20A.

Densitometric analyses indicate that macrophages treated with agrin induced a 4.02 ± 0.80 -fold increase in STAT3 expression ($n=3$, $p<0.001$ ANOVA/Tukey's HSD $p<0.05$; Figure 20B). In addition, treatment of macrophages with both agrin and LPS induced a 7.04 ± 1.70 -fold increase in STAT3 expression ($n=3$, $p<0.001$ ANOVA/Tukey's HSD $p<0.05$; Figure 20B) suggesting an additive response. The increase in STAT3 in response to LPS is not surprising since LPS has been reported to induce IL-10 as a part of a negative feedback loop. This data suggests that agrin functions upstream of IL-10 synthesis and mediates an early event that increases STAT3 intracellularly and may promote cell survival.

3.2 Neuregulin

NRG (Calvo et al., 2010) and $\alpha 7$ nAChRs (Hernandez et al., 2010) have been individually described to have neuroprotective roles in the CNS, though the two have not been investigated together. Previous work in our lab has shown that both microglia and macrophages expressed a functional erbB4 receptor, however treatments with NRG did not affect $\alpha 7$ nAChR expression in RAW264.7 macrophages though receptor phosphorylation was observed (Mencel et al., 2013). An inductive effect on $\alpha 7$ nAChR expression was only observed in BV-2 microglial cells, but agrin was unable to induce a significant increase in $\alpha 7$ nAChR expression in microglia (Mencel et al., 2013). In line with $\alpha 7$ nAChR's role in the cholinergic anti-inflammatory pathway I continued to investigate the role of NRG in

modulating this pathway in microglia by examining the synthesis of pro-inflammatory mediators (Mencel et al., 2013).

3.2.1 NRG addition results in a decrease of the pro-inflammatory cytokine TNF- α in the presence of ACh in microglia

To investigate the effects of NRG on one of the key mediators of inflammation in microglia, TNF- α , I used a commercially available TNF- α kit to analyze media harvested from BV-2 cells that had been pre-treated with 100 μ M ACh for 30 minutes prior to four hour treatments with 1nM NRG, 100ng/ml LPS, both NRG and LPS or left untreated. Similar to what was observed with macrophages, microglia treated with NRG did not exhibit an increased concentration in TNF- α when compared to untreated control (13.87 ± 3.01 pg/ml, n=7; Figure 21). As expected, challenge with LPS lead to a significant increase in the concentration of TNF- α to 403.87 ± 69.10 pg/ml (n=7, Figure 21) in microglia compared to control. In cells treated with both NRG and LPS, a significant increase in TNF- α concentration was observed (338.48 ± 64.64 pg/ml, n=7; Figure 21), however this is likely directly the result of LPS addition. The addition of ACh to microglial cultures yielded the most interesting results. BV-2 cells pre-treated with ACh and subsequently treated with NRG and LPS showed a reduced concentration of TNF- α by 32.76% to 287.80 ± 47.69 pg/ml when compared to ACh and LPS alone (428.02 ± 70.24 pg/ml, n=7, $p<0.01$, Student's *t*-test; Figure 21). These results indicate

that in the presence of ACh, NRG is a potent reducer of pro-inflammatory TNF- α (Mencel et al., 2013).

3.2.2 NRG addition results in a decrease of the pro-inflammatory cytokine IL-6 in the presence and/or absence of ACh in microglia

Another pro-inflammatory mediator modulated by α 7nAChR of the cholinergic anti-inflammatory pathway is IL-6. As such, I next investigated the role of IL-6 in response to NRG addition to microglia. Using a commercially available IL-6 ELISA kit I analyzed media harvested from treated and untreated BV-2 cells in culture, in the same manner as for TNF- α (Figure 21). Similar to TNF- α , challenging microglia with LPS lead to an increase in IL-6 concentration (12.37 ± 0.12 pg/ml, n=3) compared to control (0 pg/ml, n=3; Figure 22). The addition of NRG alone did not induce an increase in the concentration of IL-6, also similar to TNF- α . Unlike what was observed with TNF- α , the addition of NRG to LPS challenged microglia did reduce the concentration of IL-6 secreted by 75.67% (3.01 ± 0.12 pg/ml) compared to LPS challenged alone (n=3, $p < 0.05$ Student's *t*-test; Figure 22). Furthermore, NRG's effect on IL-6 synthesis is even more pronounced in microglia challenged with LPS in the presence of ACh. In this case, we see an 87.73% decrease in IL-6 between cells treated with ACh, NRG and LPS, 0.67 ± 0.17 pg/ml, compared to ACh and LPS alone, 5.46 ± 0.30 pg/ml (n=3; Figure 22). Taken together with the results observed with NRG's effects on TNF- α , these results further support an anti-inflammatory role for NRG in

microglia (Mencel et al., 2013). Curiously, NRG had no effect on IL-10 synthesis in microglia (data not shown), which could suggest that the pathways affected by NRG and agrin differ in microglia and macrophages, respectively. However, both NRG and agrin appear to function in concert with the cholinergic anti-inflammatory pathway in their respective systems.

Chapter 4

Discussion

A role for agrin in PNS and CNS synaptogenesis is well established. Within these systems agrin regulates the expression and clustering of AChRs for the efficient transmission of signals from cell-to-cell. Agrin expression in immune cells has been reported though a relationship between agrin and AChRs, like that seen in muscle cells, has not been previously investigated. In this study I report that exogenous neural agrin modulates endogenous agrin expression as well as the expression of $\alpha 7$ nAChRs, and that agrin clusters with both, in macrophages. I also show that induced endogenous agrin expression is inhibited when α -DG is blocked, suggesting that agrin mediates some of its functions through α -DG. Within the immune system agrin may function by augmenting the cholinergic anti-inflammatory pathway through DG and mediate its anti-inflammatory support in a couple of ways. In addition to augmenting $\alpha 7$ nAChRs, it appears to induce anti-inflammatory cytokine production possibly via the modulation of STAT3. Relatedly, I also demonstrate the regulation of inflammatory molecules by neuregulin. Building upon previous work completed by our lab where we show that neuregulin augments the expression of $\alpha 7$ nAChRs on microglia, the phagocytic analogs of macrophages within the CNS (Mencel et al., 2013). Significantly I also show that NRG addition to microglia results in a dramatic decrease in pro-inflammatory cytokine synthesis (Mencel et al., 2013).

This research supports a role for agrin and neuregulin as novel players in innate immunity in the peripheral and neuronal immune systems, respectively, and potentially implicates both agrin and NRG in the cholinergic anti-inflammatory pathway.

4.1 Neural agrin modulates endogenous agrin and α 7nAChR expression in macrophages

Agrin has been implicated in mediating cellular activation in immune cells by several research groups (Khan et al., 2001; Jury et al., 2007; Kabouridis et al., 2012). However, no connection has yet been identified between agrin and AChRs expressed on immune cells, though they have been examined individually (Wessler et al., 1998; 1999; Khan et al., 2001; Wessler et al., 2003; Jury et al., 2007; Neumann et al., 2007; De Rosa et al., 2009; Mazzon et al., 2011; Kabouridis et al., 2012; Mazzon et al., 2012). Agrin has been described in mediating cell activation in T cells (Khan et al., 2001; Jury et al., 2007; Kabouridis et al., 2012) and survival in monocytes (Mazzon et al., 2012), while α 7nAChRs have been demonstrated to also modulate cell activation. In lymphocytes they appear to be part of the cholinergic anti-inflammatory pathway that inhibits cytokine synthesis and T cell proliferation. Related to this, the absence of α 7nAChRs in T cells has been shown to suppress adaptive immunity (Westman et al., 2010). In macrophages, the cholinergic anti-inflammatory pathway inhibits cell activation via α 7nAChRs and can thus regulate inflammation. Given that agrin has an intricate relationship with AChRs in muscle,

regulating the expression of AChRs and contributing to the stability of AChR clusters at post-synaptic sites, and the significance of $\alpha 7\text{nAChRs}$ in regulating inflammation, an examination of the relationship of these two molecules in immune cells could be noteworthy.

Agrin is broadly and differentially expressed in various tissues. Upon the revelation that agrin is expressed in T cells (Khan et al., 2001), I was interested in determining if agrin was present in antigen presenting cells, in particular macrophages, like many other groups (Kabouridis et al., 2012; Mazzon et al., 2012). As it turned out, of peripheral blood mononuclear cells (PBMCs), agrin is predominantly expressed by the myeloid cell lineage that differentiates into monocytes/macrophages (Mazzon et al., 2012). Agrin expressed in T cells, has been reported to be in the transmembrane form and does not contain the Z insert site (Khan et al., 2001). In T cells, this isoform has been demonstrated to play a role in cell activation; it functions to decrease the threshold of activation by clustering T cell receptors (Khan et al., 2001). T cells are crucial to the development of adaptive immune responses and given the interplay between innate and adaptive immunity I wondered if agrin played a similar role in the development of innate responses and in antigen-presenting cells in particular. Given this, I elected to investigate the function of agrin in macrophages.

Macrophages are primary responders in the innate immune response and function to eradicate infection by the means of inflammation. Using an immortalized murine macrophage cell line RAW264.7 I showed that agrin is expressed in these cells (Figure 7). The agrin expressed here is likely the transmembrane form, though other isoforms cannot be excluded. In T cells, the addition of a truncated agrin isoform, termed neural agrin, induced

the activation of these cells. This induction was demonstrated by the deglycosylation of Tm-agrin and the clustering of various receptors and molecules (Khan et al., 2001). Similarly, I looked for agrin induced cell activation in macrophages in conjunction with treatments with LPS, a potent activator of macrophages and inducer of inflammation, for a means of comparison. A significant induced increase in the expression of endogenous agrin in response to agrin but not LPS was observed independently (Figure 8). Furthermore, various concentrations of agrin can induce an increase in the expression of endogenous agrin in macrophages (Figure 9) mirroring it's effects in muscle cells, where endogenous agrin expression is upregulated by the addition of neurally-derived agrin (Lieth and Fallon, 1993). These initial results raised some interesting questions about the role of agrin in inflammation, as we observed a different phenotype in endogenous agrin expression induced by a known inflammatory mediator, LPS, and by agrin. Curiously, agrin has been shown to induce capping of CD14, the co-receptor of TLR4 for LPS, and cluster MHC, involved in antigen presentation in macrophages, which would suggest a pro-inflammatory role for agrin (Kabouridis et al., 2012). However, we observed that longer treatments with agrin (24 hours) did not result in cell death, as exemplified by LPS treatment (Raschke et al., 1978). This agrees with a recent study that shows that agrin deficient macrophages are defective in mediating phagocytosis for pathogen eradication and exhibit enhanced apoptosis (Mazzon et al., 2012), suggesting a role for agrin in macrophage survival. In T cells, the addition of interferon (IFN) α induces increased agrin expression with this expression coinciding with an increase in anti-inflammatory IL-10 synthesis (Jury et al., 2007). Finally, agrin was not

shown to activate NF- κ B, which is an effector of LPS function that results in inflammation (Jury et al., 2007). Taken together these results do not support an inflammatory role for agrin and may in fact imply the contrary.

The expression of α 7nAChR on macrophages and their involvement in regulating inflammation (Wang et al., 2003a) lead us to investigate the relationship between agrin and α 7nAChRs in these cells. Cholinergic neurons, such as the vagus nerve, regulate inflammation by secreting ACh that functions on various immune cells in the periphery, including macrophages (Borovikova et al., 2000). All cholinergic neurons secrete agrin as well, so it is not unlikely that neural agrin can mediate its effects on macrophages. Agrin signaling at the NMJ, through the convergence of multiple pathways, induces the expression of AChRs among other post-synaptic proteins (Lacazette et al., 2003). I demonstrated that agrin could also induce an increase in the expression of α 7nAChRs in macrophages (Figure 12). This suggests a regulatory role for agrin in inflammation by modulating the cholinergic anti-inflammatory pathway. The presence of AChRs is required for agrin-induced clustering of post-synaptic molecules in muscle (Marangi, 2001) and for the initiation of adaptive immune response in T cells (Westman et al., 2010). Agrin has been demonstrated to cluster various molecules on immune cells, however α 7nAChR clustering has not been examined. In muscle, AChRs are required for the clustering of other post-synaptic molecules in response to agrin (Marangi, 2001). Our results demonstrate that agrin regulates α 7nAChR clusters on macrophages (Figure 14). Furthermore, these clusters co-localize with endogenous agrin (Figure 15) and both agrin and α 7nAChRs interact with the agrin receptor, α -DG (Figure

16). These events are analogous to what occurs in muscle where endogenously expressed agrin co-localizes with the majority of AChRs (Lieth and Fallon, 1993). The clustering of AChRs is thus crucial for efficient communication between cells, regardless of the type of cell.

4.2 Possible mechanisms of agrin action in macrophages

In macrophages, activation of NF-κB leads to inflammation by inducing synthesis of pro-inflammatory cytokines such as TNF- α . Interestingly, the transcription factor NF-κB has been shown to regulate AChR clustering in muscle through increased *rapsyn* promoter activity resulting from the upregulation of RelA/p65 (Wang et al., 2010). As such NF-κB appears to play a role in the regulation of rapsyn expression. LPS and TNF- α are potent inducers of NF-κB activity and recently a number of neuronal factors have also been shown to activate this transcription though it appears that agrin does not activate NF-κB (Wang et al., 2010). We indirectly support that agrin does not activate NF-κB in macrophages as agrin does not appear to induce the synthesis of pro-inflammatory mediators in these cells (Figure 17 and 18). It is possible that in macrophages, NF-κB also influences AChR clustering and through the *rapsyn* promotor modulates the expression of agrin. At this point it is unknown if rapsyn or a rapsyn-like protein is expressed in macrophages but if expressed it might explain the decreased expression of endogenous agrin induced by ACh (Figure 13) through the inhibition of NF-κB. Similar to in muscle, agrin signaling in these cells does not appear to

activate NF-κB, if the lack of TNF-α is indicative (Figure 17). It is possible that more than one pathway in macrophages regulates the clustering of AChRs. In muscle and macrophages, the ERK/MAPK pathway has been implicated in agrin signaling, though it has thus far been shown to have at least a minor, albeit negative role, in modulating clustering of AChRs in muscle (Rimer, 2010). In T cells the ERK pathway is activated by agrin cross-linking and leads to cytoskeletal re-organization, but stimulation with neural agrin directly has not been examined (Jury et al., 2007). The ERK/MAPK pathway makes for a likely candidate in mediating clustering in macrophages, but it is unclear if in these cells the effect would be positive or negative. In addition to this pathway, PI3K is also a strong candidate for being involved in mediating clustering events in macrophages. Agrin signaling has been shown to activate PI3K pathways in macrophages (Kabouridis et al., 2012) and muscle (Nizhynska et al., 2007) as well. Though PI3K is primarily reported in activating cell survival pathways, it has also been implicated in agrin-induced AChR clustering in muscle by regulating Rac and cdc42, involved in cytoskeletal re-organization (Nizhynska et al., 2007). Furthermore, PI3K inhibitors have the effect of inhibiting full-size AChR cluster formation by agrin and reducing AChR phosphorylation (Nizhynska et al., 2007).

Several studies have proposed α-DG as the agrin receptor in immune cells (Zhang et al., 2006; Kabouridis et al., 2012; Mazzon et al., 2012). DG in muscle co-localizes with AChRs via rapsyn and has also been previously implicated in stabilizing AChR clusters (Apel et al., 1995). Interestingly, it has been recently proposed that agrin mediates survival signaling through α-DG, as α-DG has been shown to co-localize with Grb2 in monocytes

(Mazzon et al., 2012), as it does in muscle (Yang et al., 1995). In monocytes, signaling through α -DG activates PI3K signaling and blocked α -DG-agrin signaling enhances apoptosis in these cells (Mazzon et al., 2012). Similarly in muscle, disruption of α -DG signaling has been shown to disrupt PI3K signaling (Langenbach and Rando, 2002). Agrin has three major receptors in the CNS and the PNS: α 3NKA, LRP4 and DG. These three receptors have been shown to be expressed in immune cells as well, with the highest proportion being α 3NKA (Kabouridis et al., 2012). In light of the work completed by Mazzon and colleagues (2012), we investigated agrin-DG function. Agrin binds DG with great affinity at the NMJ (Bowe et al., 1994; Campanelli et al., 1994; Fallon and Hall, 1994; Gee et al., 1994; Sugiyama et al., 1994) and due to this affinity, DG seemed like a good candidate for an agrin receptor on macrophages that might mediate agrin's functions. My results indicate that blocking the function of α -DG also inhibits an increase in endogenous agrin expressed in macrophages by neural agrin (Figure 11). Taken with previous work indicating that blocking α -DG also disrupts PI3K pathways (Mazzon et al., 2012) this further suggests α -DG as at least one immune regulatory receptor in macrophages through which agrin signaling may be self-regulatory. Other receptors for agrin cannot be excluded in playing a role in regulating inflammation in macrophages. Although both α 3NKA and Lrp4 have been described to be present on macrophages, no role has yet been determined (Kabouridis et al., 2012). In T cells, it has also been suggested that Tm-agrin may function as a receptor on its own (Jury et al., 2007). It is possible that this is also true in macrophages,

however my results only address endogenously expressed total agrin protein and it is unclear if the agrin expressed in these macrophages is Tm-agrin. Finally, the anti- α -DG antibody used in blocking experiments has an IgM heavy chain isotype, and thus is quite large. Because α -DG has been shown to co-localize with both endogenous agrin and α 7nAChRs (Figure 16), it is possible that endogenous and α 7nAChRs get blocked as well by this anti- α -DG antibody.

Curiously, blocking of DG in macrophages subsequently treated with LPS also yielded a similar phenotype to that seen with agrin (Figure 11). It is possible that DG and the LPS receptor, CD14, interact with each other and thus the blocking of DG also blocks CD14. This also could explain reports indicating that neural agrin induces capping of CD14 in PBMCs (Kabouridis et al., 2012).

At the NMJ, ACh has a declustering and destabilizing effect on AChRs and tends to negate agrin's effects via a Cdk5 mechanism (Lin et al., 2005; Misgeld et al., 2005). Stabilized clusters that are agrin-induced have a longer half-life on muscle than destabilized clusters, which are more likely to get internalized and recycled (Lin et al., 2005; Misgeld et al., 2005). This destabilizing effect is prominent in agrin isoforms lacking the Z insert (Misgeld et al., 2005). It is unknown if ACh would also counteract agrin-induced expression of endogenous agrin in muscle. However, this event is observed in macrophages that were subjected to treatment with ACh (Figure 13). ACh is considered to activate immune cells in a non-classical manner (De Rosa et al., 2009) and this ACh-induced cell activation does not induce an inflammatory response. Similarly, agrin-induced cell activation is not indicative of

a pro-inflammatory phenotype as a result of surface receptor clustering. The initiated signaling pathway may converge with ACh initiated signaling pathways. Though early events appear antagonistic, they could still function in concert. A balance of events mediated by agrin and ACh may allow for proper immune response within a cell by achieving homeostasis with complex regulatory mechanisms that result in a similar purpose when confronted with inflammatory stimuli, namely anti-inflammatory regulation.

4.3 Agrin may have a primarily anti-inflammatory role in macrophages

The ability of agrin to induce endogenous agrin and $\alpha 7\text{nAChR}$ expression suggests that agrin in macrophages may influence the cholinergic anti-inflammatory pathway that mediates the regulation of inflammation. The effect of agrin signaling in macrophages has recently been investigated with my results indicating a primarily anti-inflammatory role for agrin. Initial experiments examined the effect of agrin on pro-inflammatory cytokines TNF- α and IL-6 synthesis in macrophages as these cytokines are directly and indirectly inhibited by the cholinergic anti-inflammatory pathway. I did not see an induction of the pro-inflammatory cytokine TNF- α (Figure 17) to any significant level after four hours in treatment with agrin when compared to LPS, which is a potent inducer of inflammatory cytokines. However, my results do indicate that agrin reduces IL-6 by 57% in LPS challenged macrophages (Figure 18). Recently, Kabouridis and colleagues (2012) suggested a pro-inflammatory role for agrin in monocytes by examining TNF- α and IL-6. However, in this case, agrin only induced an increase in these cytokines in the presence of polyinosine-polycytidylic acid (poly-IC), which

activates a subgroup of TLRs (Kabouridis et al., 2012). The induction of TNF- α and IL-6 is, in that instance, likely the result of poly-IC itself (Kabouridis et al., 2012). The addition of agrin also augmented anti-inflammatory cytokine IL-10 secretion in RAW264.7 macrophages (Figure 19), indicated by a 15-fold increase in agrin-treated macrophages. This is in agreement with a study in Lupus T cells that demonstrated that over-expression of agrin lead to augmented IL-10 expression (Jury et al., 2007). This is likely due to modulation of STAT3 expression by agrin (Figure 20). The Jak2-STAT3 pathway is one mechanism by which the cholinergic anti-inflammatory pathway functions (de Jonge et al., 2005). STAT3 is an anti-inflammatory regulator that has been implicated in the inhibition of NF- κ B-regulated pro-inflammatory cytokines and the synthesis of IL-10 via a positive feedback pathway. STAT3 can be activated through a few mechanisms: LPS-induced p38 MAPK signaling, IL-10 receptor signaling, and ACh-induced Jak2 signaling. In monocytes, agrin has been reported to increase internal Ca²⁺ flux, transiently activating Akt/GSK3 of the PI3K pathway (Kabouridis et al., 2012). Interestingly, signaling through the PI3K-Akt-GSK3 pathway has been implicated in suppressing inflammation by augmenting IL-10 inducible genes (Antoniv and Ivashkiv, 2011). Further supporting an anti-inflammatory role for agrin are experiments completed with agrin-deficient monocytes, which exhibit low survival and are defective in phagocytosis (Mazzon et al., 2012). Agrin has also been shown to signal through α -DG-Grb2 to activate ERK/MAPK pathways. It is unclear if agrin also activate p38 α MAPK which has anti-inflammatory roles consistent with STAT3-IL-10 functions (Bode et al., 2012). Finally, IFN α has also been reported to induce agrin expression in T cells

(Jury et al., 2007) via STAT3 (Ziegler-Heitbrock et al., 2003). These results taken together support claims for agrin as a novel player in innate immunity.

4.4 NRG fulfills similar roles to agrin in the neuronal immune system of the CNS

Anticipating functional parallels, I also investigated potential roles for agrin and NRG on microglia, the macrophages of the CNS. The CNS is an immune privileged site in which the BBB separates its own immune system from the periphery. Previous work completed by our lab has indicated that microglia expressed erbB4 receptors and that they form functional receptors in microglia capable of being phosphorylated by NRG (Mencel et al., 2013). NRG was also able to induce an increase in the expression of $\alpha 7$ AChRs in microglia, however when NRG is added to macrophages we do not see a significant increase (Mencel et al., 2013). Agrin addition to microglia had no significant effect on $\alpha 7$ nAChR expression (data not shown). This suggests that agrin and NRG may have complementary roles in the peripheral and neuronal immune systems. Being that $\alpha 7$ nAChRs are the most abundant form of acetylcholine receptors in the CNS, crucial to the cholinergic anti-inflammatory pathway, and NRG can regulate these receptors, we focused our attention on NRG-induced modulation of the pro-inflammatory cytokines TNF- α and IL-6 synthesis in microglia (Mencel et al., 2013). Though we did not see NRG modulate IL-10, an anti-inflammatory cytokine, our results do indicate that NRG may play an anti-inflammatory role by reducing TNF- α and IL-

6 levels. NRG reduced TNF- α levels in microglia challenged with LPS by 33%, though only in the presence of ACh (Figure 21). Additionally, NRG alone also reduced IL-6 levels in microglia challenged with LPS by 76% (Figure 22). Taken together, these results imply an anti-inflammatory role for NRG on microglia, different from that seen with agrin on macrophages, wherein it inhibits pro-inflammatory cytokine synthesis.

4.5 Agrin and NRG: Different functions, similar roles, in the periphery and the brain

Inflammation is an essential part of the innate immune system, necessary for proper infection eradication and wound healing. The dysregulation of aspects of the inflammatory response is the basis of many chronic diseases. The augmentation of the cholinergic anti-inflammatory pathway and exploitation of the molecules of this system can potentially prevent some unnecessary damage that results from prolonged exposure to pro-inflammatory mediators. The modulation of α 7nAChRs can thus be considered a therapeutic means of providing homeostasis to an unbalanced response and α 7nAChR-mediated targets are already under investigation (Bencherif et al., 2011). Inflammation has been implicated in many chronic diseases, including (but not limited to) arthritis, Alzheimer's, Chron's, diabetes, atherosclerosis, psoriasis, asthma, cystic fibrosis, ulcerative colitis and cancer (Reviewed in Bencherif et al., 2011). In these diseases, agrin and NRG might prove to be new targets for

anti-inflammatory therapeutics. Agrin and NRG mediate survival pathways by augmenting the cholinergic anti-inflammatory pathway in their respective systems.

Agrin demonstrates an anti-inflammatory role in macrophages and can function in concert with the cholinergic anti-inflammatory pathway in two ways: through early STAT3 induction and later augmentation of α 7nAChRs and anti-inflammatory IL-10. Though agrin does not work in concert with ACh, it could stabilize α 7nAChR clusters necessary for mediating signal transduction from ACh and thereby potentiate the cholinergic anti-inflammatory response.

Similarly, in microglia, NRG augments α 7nAChR expression and this coincides with a decrease in pro-inflammatory cytokines TNF- α and IL-6, thus also exhibiting an anti-inflammatory role. Here, NRG appears to function in concert with ACh and ameliorates long-term effects of inflammation, though how this may be occurring needs further investigation.

The results presented in this thesis offer a new perspective into the regulation of inflammation through the modulation of the cholinergic anti-inflammatory pathway. Here I implicate agrin and NRG as novel players in innate immune responses in the periphery and CNS capable of augmenting cytokine synthesis. Though agrin and NRG have slightly different functions in macrophages and microglia, respectively, the end result is the same: an anti-inflammatory one.

4.6 Future Directions

It is evident from this work that agrin and NRG, two neurotrophic molecules, can function in innate immune response by promoting an anti-inflammatory phenotype in immune cells through the augmentation of components of the cholinergic anti-inflammatory pathway and cytokine synthesis. Although some insight into the roles of these molecules can be gained from previous studies completed in the PNS and CNS, still a lot of avenues need to be investigated into how these molecules function.

One particular binding partner of interest and mediator of clustering of AChRs at the NMJ, mentioned in the discussion, is rapsyn. Thus far it is unknown whether rapsyn or a rapsyn-like molecule exists in immune cells, but because of the presence of DG and AChRs in these cells makes it a worthwhile molecule to investigate. Furthermore, a previous report has indicated that another agrin receptor, LRP4 is expressed in immune cells (Kabouridis et al., 2012). LRP4 is known to indirectly link AChRs through rapsyn at the NMJ thus making it a potentially interesting molecule to investigate. In particular, if present, how might agrin regulate its expression? Does it bind to a MuSK-like receptor tyrosine kinase, and how might this receptor complex influence an immune response? A MuSK-like protein could also regulate AChR phosphorylation providing an additional regulatory mechanism for the cholinergic anti-inflammatory pathway.

In line with our previous work, the regulation of AChR synthesis in immune cells might also be an interesting avenue of investigation. Given that the PI3K pathway has been implicated in activating the N-box promotor in muscle, the regulation of a similar promotor,

perhaps an E-box promoter (for α -subunit AChRs, if present) or the presence of a *rapsyn* promoter would give further insight into just how agrin and NRG mediate their effects.

Regardless of the particular focus, the results presented here have revealed new roles and research opportunities for agrin and NRG. The study of the function of these molecules in inflammation might hold the key to the amelioration of any number of inflammation-based diseases.

Figures

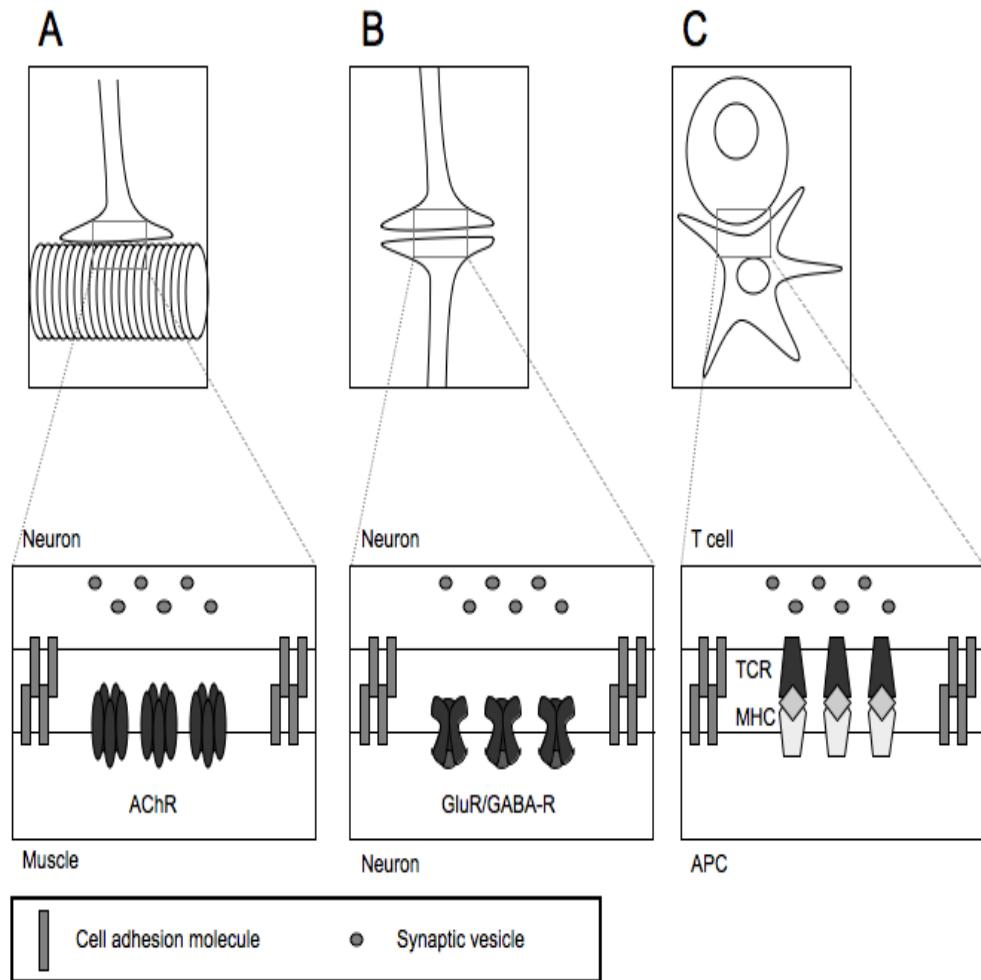


Figure 1. Synapses in the PNS, CNS and immune system are a stable connection between cells.

These junctions allow for efficient chemical communication and can evoke complex responses in participating cells. Synapses, regardless of the tissues involved, are very similar in structure and function. Pre-synaptic terminals contain synaptic vesicles containing chemical mediators. Cell adhesion molecules maintain contact and concentrate chemical mediators in the synaptic cleft, between cells, allowing for efficient communication. Post-

synaptic terminals typically contain clustered receptors for the appropriate chemical messengers. Panel A shows a NMJ from the peripheral nervous system. The presynaptic terminal here is a motor neuron and the postsynaptic terminal is the muscle fiber. In the presynaptic terminal, synaptic vesicles contain the neurotransmitter acetylcholine. The postsynaptic terminal is a muscle fiber. (B) In the central nervous system, interneuronal synapses are prevalent and can be segregated into inhibitory (GABAergic) and excitatory synapses (glutameric), which are dependent on the channels that are opened as the result of ligand binding to their specific receptors. Synaptic vesicles in the pre-synaptic terminal contain GABA in inhibitory synapses, which bind GABA-R in the post-synaptic terminal. Synaptic vesicles in the pre-synaptic terminal contain AMPA or NMDA in excitatory synapses, which bind glutamate receptors (Glu-R) in the post-synaptic terminal. Panel C depicts an immune synapse formed between an antigen-presenting cell (APC) and a T cell, but synapse between many types of immune cells can form. In this case, the antigen presented by the APC via its multihistocompatibility complex (MHC) can create a response in the T cell, through its T cell receptor (TCR) that is antigen-specific. Synaptic vesicles contain cytokines that can function on either cell and thus the role of pre- or post-synaptic terminal is dynamic in immune synapses.

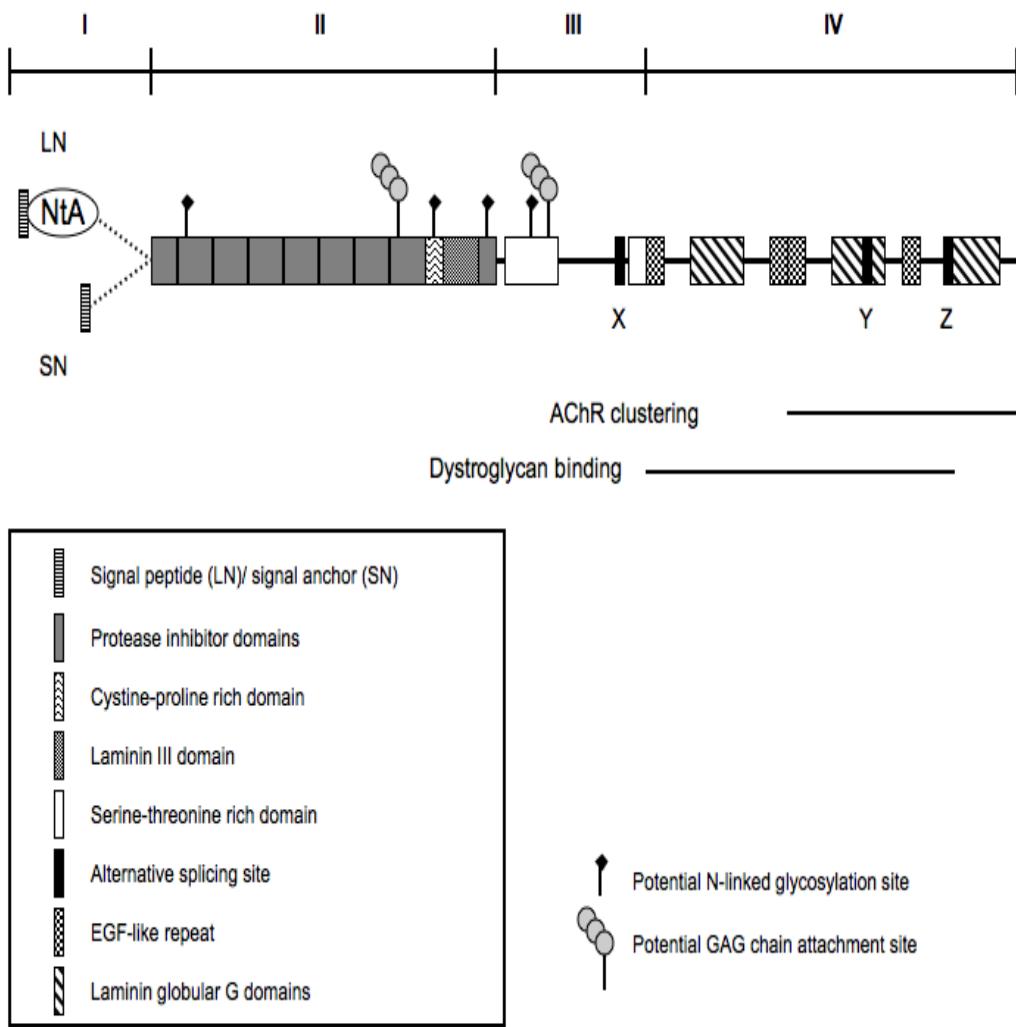


Figure 2. Agrin and its domains.

This schematic representation of agrin depicts the sites of alternative splicing, structural domains, glycosylation sites and potential binding sites of important partners. Two isoforms of agrin are the result of alternative splicing at the N-terminus and these transmembrane and secreted forms of agrin can be further spliced at the Y and Z sites on the C-terminus of the agrin molecule. A 95 kDa fragment of agrin that spans from EGF1 to the C-terminus fully retains agrin's clustering ability at the highest potency. Amino acid inserts at the Z site yield an 'active' agrin isoform, termed neural agrin, and that is essential for agrin-induced clustering of AChRs on myotubes. Muscle agrin is the result of an 4 or 0 amino acid insert at the Y site and neural agrin is the result of an 8 amino acid insert at the Z site.

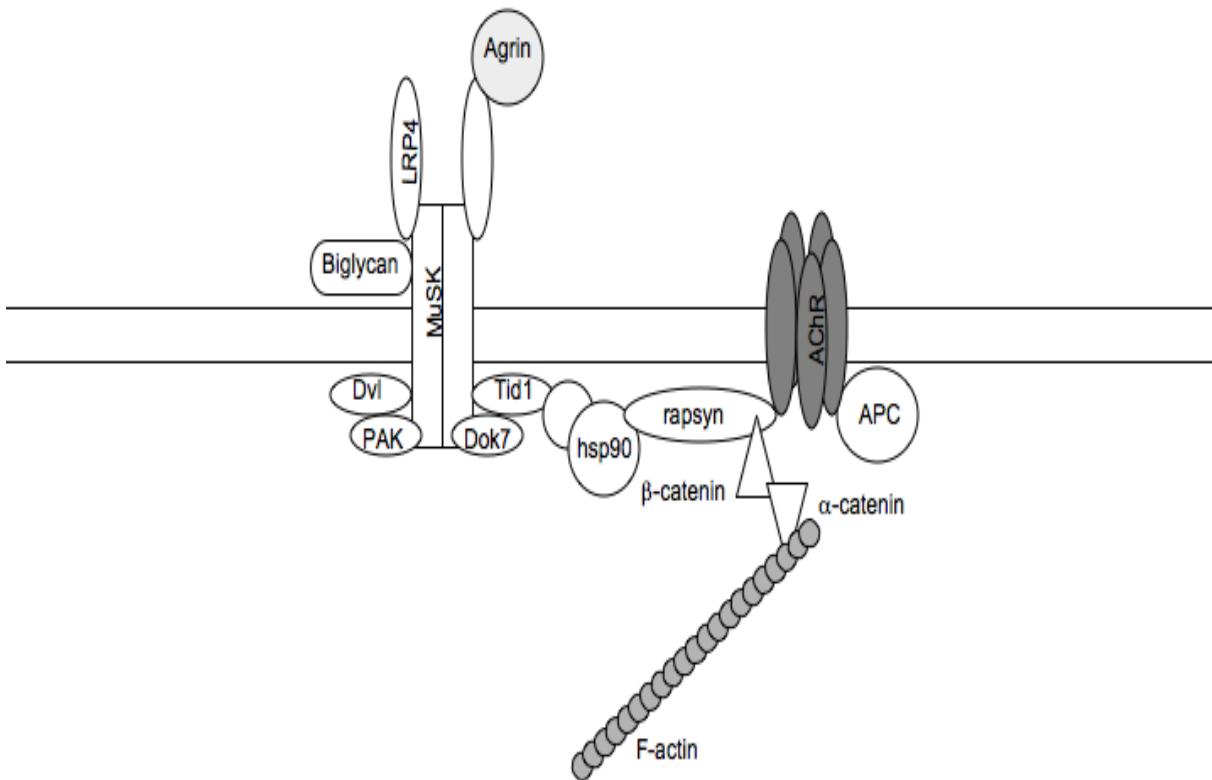


Figure 3. A model of the agrin-MuSK complex at the NMJ.

Agrin signaling through MuSK occurs through the binding of LRP4. MuSK is a receptor tyrosine kinase (RTK) where phosphorylation is mediated by adaptor proteins Dok7 and Tid1. Tid1 partially activates MuSK in the absence of agrin, and enhances the binding of LRP4 to agrin. Tid1 regulates the binding of Dok7 to MuSK and stabilizes AChR clusters through hsp70 and hsp90 bound to rapsyn. Rapsyn links AChRs to the actin cytoskeleton via α - and β -catenin. Rapsyn can also mediate the binding of DGC to AChRs (see Figure 4) though whether rapsyn can also link AChRs to the cytoskeleton has not been directly implied. Dvl and PAK also bind to MuSK and MuSK phosphorylation activates Tid1 and Dvl, which regulate Rho GTPases, Rac1/RhoA/cdc42 (not shown) and thus regulates AChR clustering.

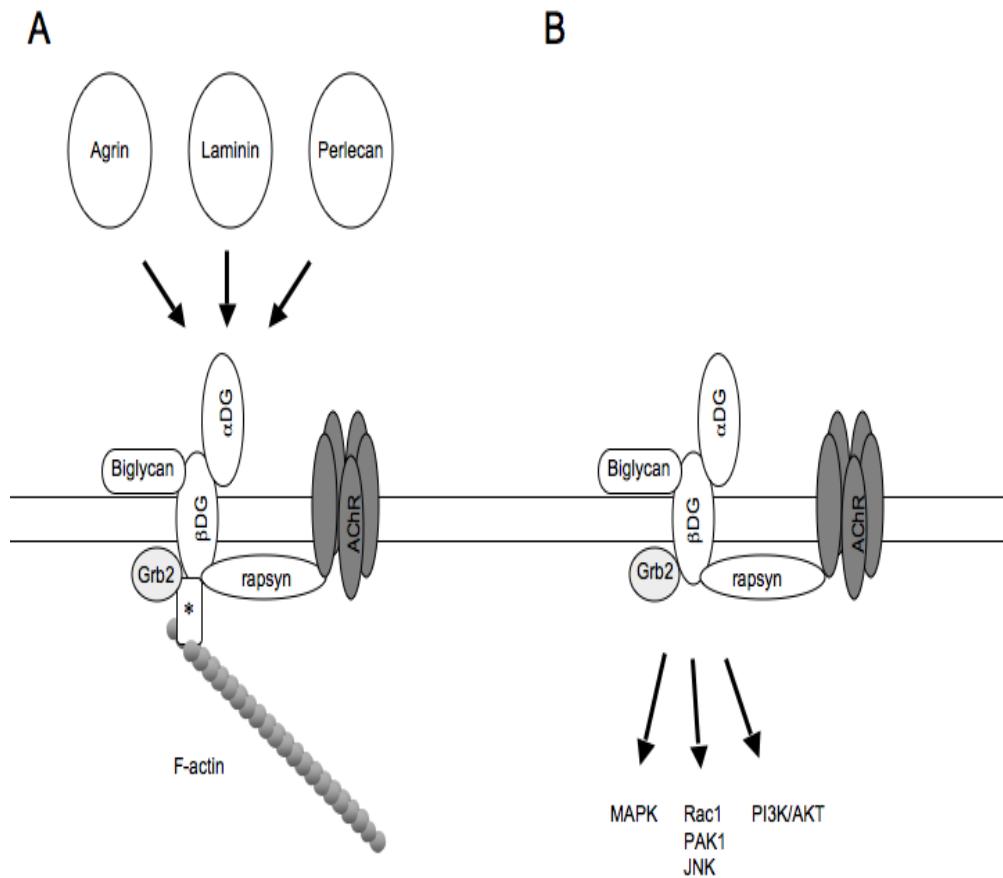


Figure 4. A simplified model of the dystroglycan complex (DGC) at the NMJ.

This complex is part of a larger dystrophin-associated protein (DAP) complex present in muscle. In panel A, we see that the DGC is composed of α -dystroglycan (α -DG; peripheral membrane protein) and β -dystroglycan (β -DG; transmembrane protein). Agrin, laminin and perlecan are extracellular matrix protein able to bind α -DG. Dystrophin or utrophin (*), the homolog of dystrophin, bind to the C-terminus of β -DG and this connects the DGC to the cytoskeleton through the association of filamentous actin (F-actin). Although not considered part of the original complex we also show AChRs interacting with the DGC through rapsyn. Biglycan (extracellularly) and the signaling molecule Grb2 (intracellularly) also interact with β -DG. In panel B, we show possible downstream signaling pathways that have been associated with the DGC. These pathways may be mediated by Grb2 and include: MAPK, PI3K/AKT (survival) and Rac1/PAK1/JNK (cell growth and cytoskeletal reorganization). Molecules not shown here that also interact with the dystroglycan and considered part of the DAP complex include the membrane proteins sarcoglycan, sarcospan, and intracellular proteins dystrobrevin and syntrophin.

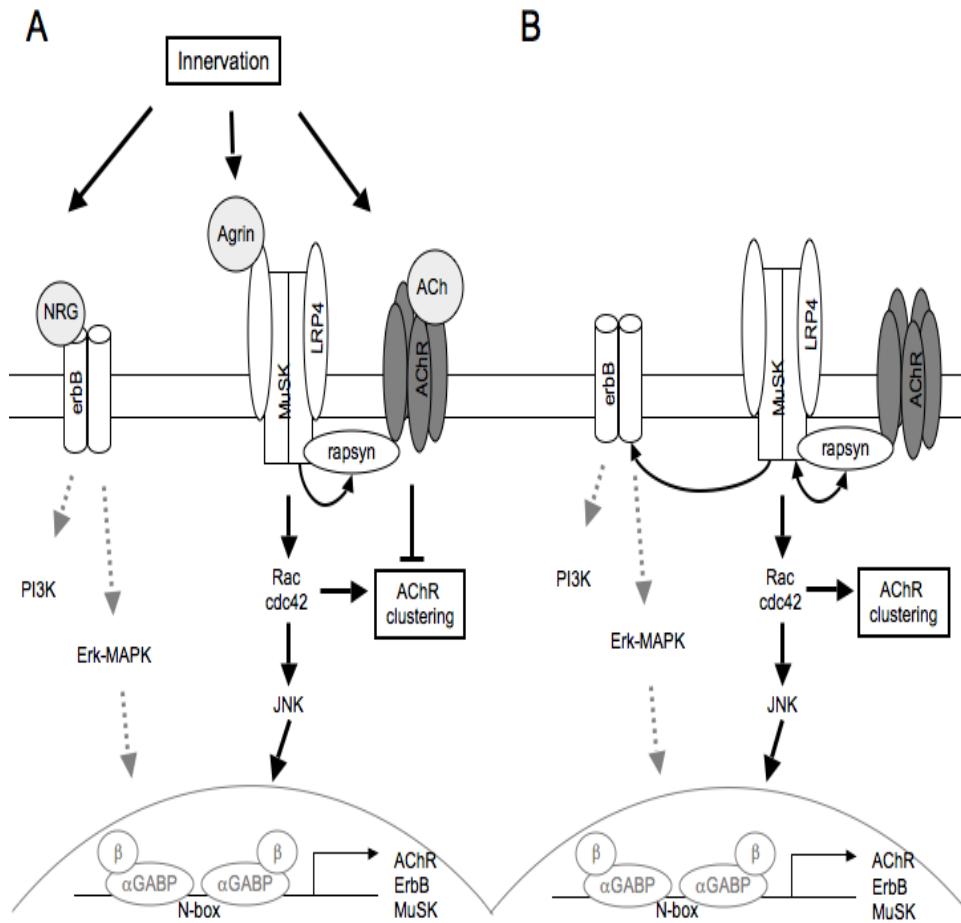


Figure 5. MuSK signaling is essential for post-synaptic specializations on muscle.

Panel A shows that in the presence of innervation, neuronal signals that include agrin, neuregulin (NRG) and acetylcholine (ACh). NRG binding of erbB receptors and agrin binding of MuSK leads to phosphorylation of these receptors. MuSK phosphorylation activates rapsyn and downstream signaling pathways include Rac and cdc42 (AChR clustering) and JNK. ErbB phosphorylations can activate PI3K and MAPK signaling pathways. Activation of MAPK signaling and JNK converge upon the activation of nuclear GA-binding protein (GABP) which binds an N-box promoter sequence and leads to the transcription of post-synaptic molecules: ErbB, MuSK and AChRs. In innervated muscle the AChR aggregates are more refined in synaptic regions and this can be attributed to muscle activity initiated by ACh, diminishing AChRs extra-synaptically (not shown). In panel B we see that in the absence of innervation MuSK phosphorylated by rapsyn can activate erbB and rapsyn sustaining the same pathways seen during innervation. Grey regions indicate original views regarding NRG and agrin co-operation that have been since challenged or considered dispensable for synaptogenesis.

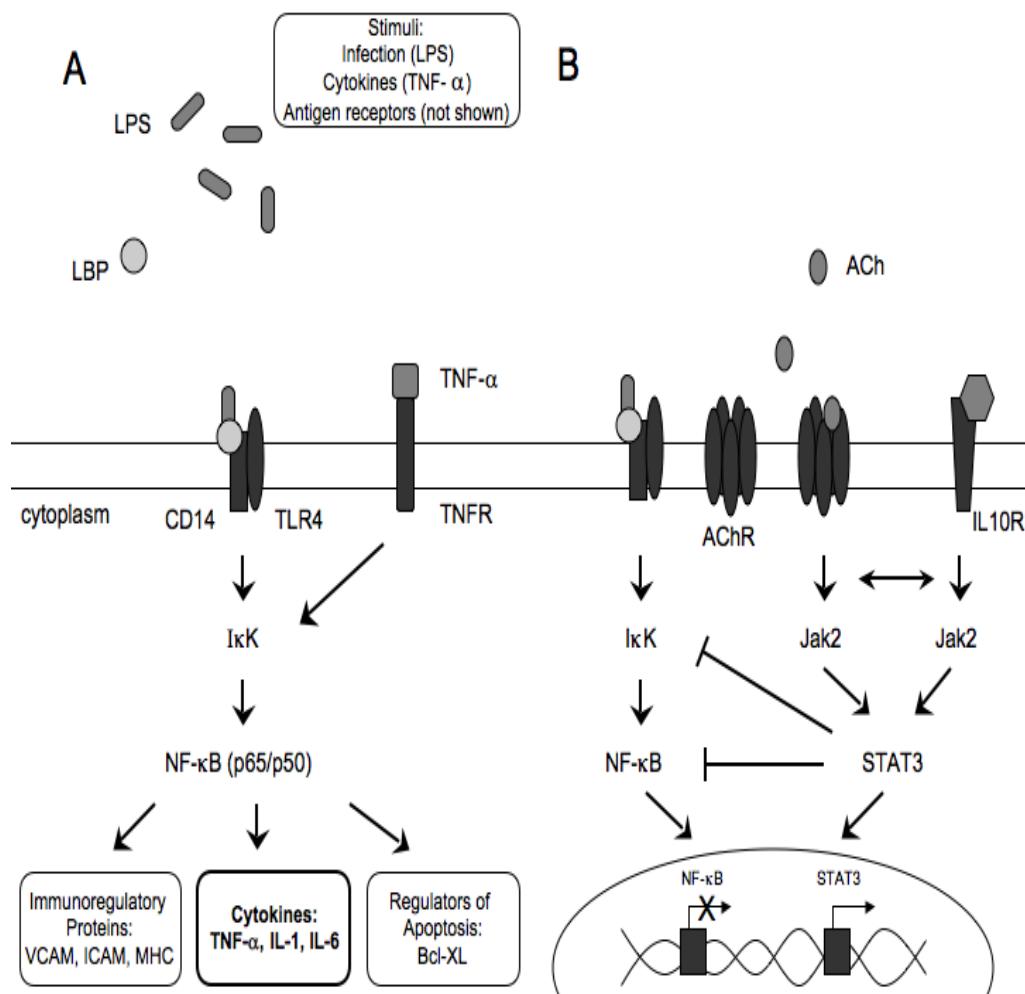


Figure 6. Inflammation is regulated via the cholinergic anti-inflammatory pathway in macrophages.

In panel A, we see the canonical inflammatory response initiated by lipopolysaccharide (LPS) binding of Toll-like receptor 4 (TLR4) and CD14 via LPS-binding protein (LBP). Signaling through TLR4 recruits adaptor molecules (not shown) that lead to the activation of IKK complex, and inhibitory complex that then frees NF- κ B for nuclear translocation and transcriptional activation of pro-inflammatory genes. NF- κ B is responsible for the expression of immunoregulatory proteins that promote cell adhesion (VCAM and ICAM) and antigen presentation (MHC), pro-inflammatory cytokines that function in an autocrine/paracrine manner (TNF- α , IL-1, and IL-6), as well as apoptotic regulators (i.e. Bcl-XL). TNF- α also further propagates this reaction by activating IKK through signaling of the tumor necrosis factor receptor (TNFR), thus sustaining an inflammatory response. In panel B we see the cholinergic anti-inflammatory pathway that is reliant on ACh secreted by the vagus nerve (not shown), which innervates the peripheral organs, such as the liver and spleen. These

organs are afflicted if localized inflammation turns systemic and leads to septic shock, which results in organ failure and fatality. The efferent arm of the vagus nerve secretes acetylcholine in response to the presence of proinflammatory cytokines and endotoxins detected by the afferent arm of the vagus nerve (not shown). Endogenous acetylcholine, an agonist, binds the α 7nAChR (AChR) present on macrophages, and inhibit the production and release of pro-inflammatory cytokines by two possible mechanisms, where activation of α 7nAChR can 1) directly inhibit the nuclear translocation of NF- κ B, and/or 2) initiate the Jak2/STAT3 pathway in which Jak2 is recruited to the α 7nAChR, becomes phosphorylated and then phosphorylates STAT3. STAT3 dimerizes and is translocated to the nucleus and initiates transcription of anti-inflammatory genes.

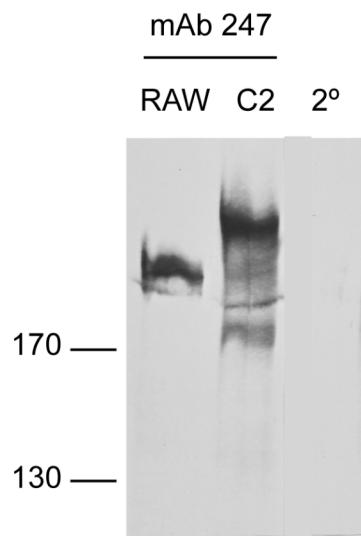


Figure 7. Agrin is endogenously expressed in the RAW264.7 macrophage cell line.

A representative western blot for agrin in RAW264.7 macrophages (RAW) and C2C12 myotubes (C2) demonstrates that agrin is expressed in macrophages, similar to muscle, although it appears to be less glycosylated and not as highly expressed. Molecular mass markers are shown to the left in kDa.

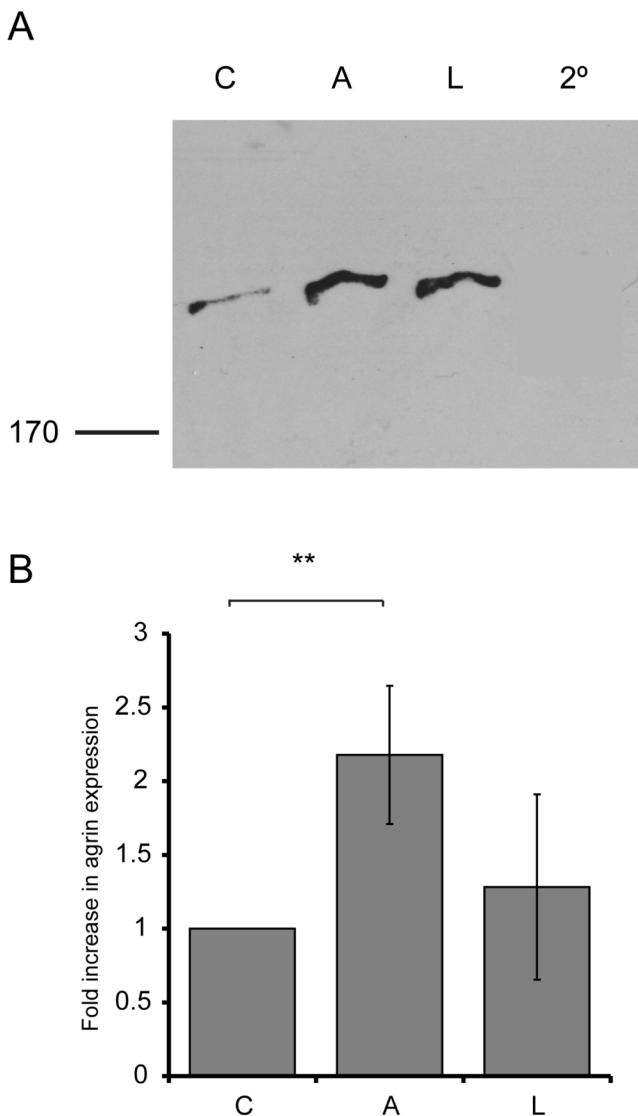


Figure 8. Agrin induces increased endogenous agrin expression in macrophages.

A representative western blot for agrin in RAW264.7 cells following treatment with agrin (A; 500pM) or LPS (L; 0.1 μ g/ml) over four hours is shown in panel A. Control cells were left untreated for four hours before extraction (C). Molecular mass markers are shown to the left in kDa. Expression levels of agrin were quantified via densitometry and are represented as averages (mean \pm SD) in panel B. Agrin induces the expression of endogenous agrin 2.18 ± 0.47 -fold after four hours compared to basal levels observed in the untreated control **(n=5, Student's *t*-test $p<0.001$). LPS addition resulted in 1.28 ± 0.63 -fold increase in endogenous agrin expression, though this is not significant.

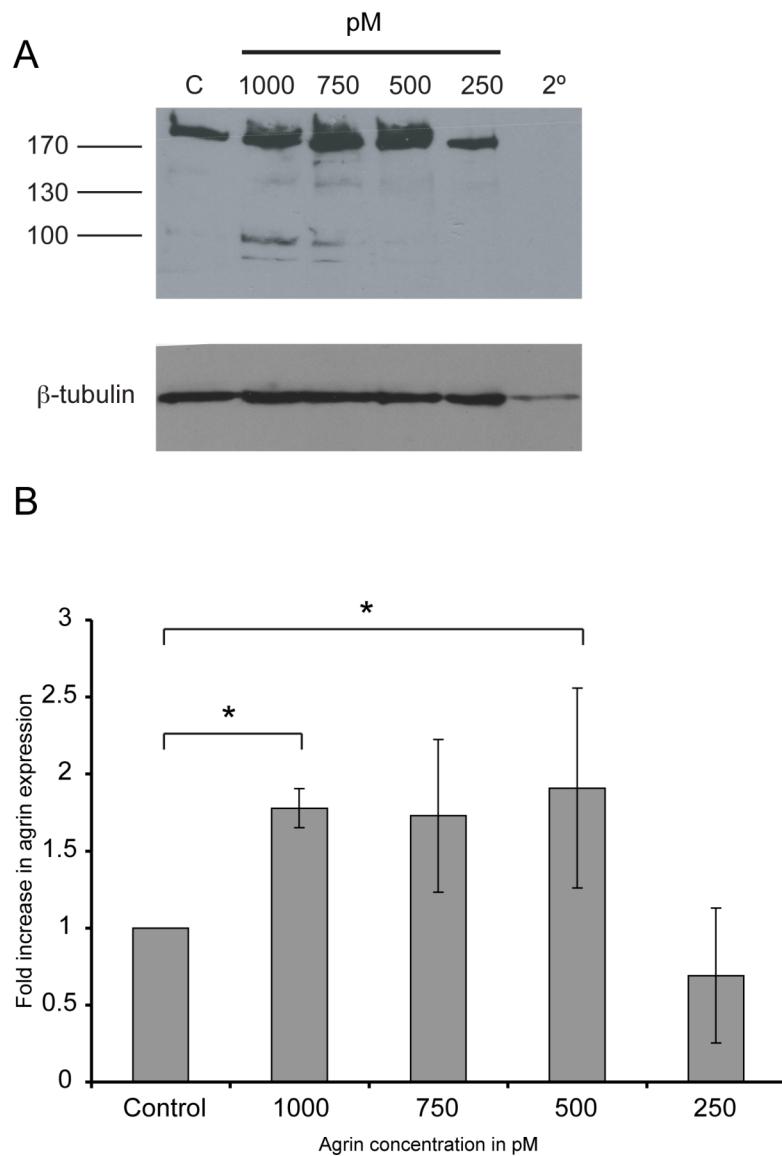


Figure 9. Macrophages are responsive to various concentrations of agrin.

RAW264.7 macrophage cells were treated for four hours with 1000pM, 750pM, 500pM and 250pM of agrin, or left untreated (control). A representative western blot for endogenous agrin expression is shown in panel A. Molecular mass markers are shown to the left in kDa. Densitometric analysis of agrin expression is displayed in panel B and represents the averages from three trials (mean \pm SD). Treatment of macrophages with 500pM induces the highest level of endogenous agrin expression (1.91 ± 0.65 fold, n=3), a value consistent with our earlier experiments *($p<0.05$ ANOVA/Fisher's LSD $p<0.05$).

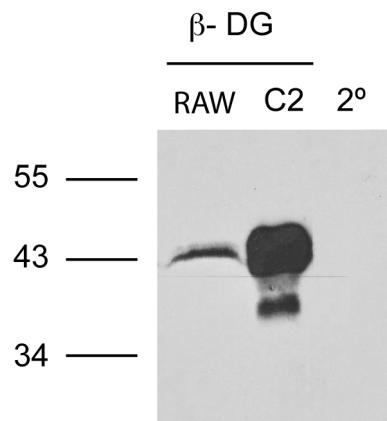


Figure 10. β -DG is expressed by RAW264.7 macrophages.

A representative western blot for β -DG in RAW264.7 macrophages (RAW) and C2C12 myotubes (C2) demonstrates that β -DG is expressed in macrophages, similar to muscle, although it appears to be not as highly expressed. The presence of β -DG indicates that α -DG must also be expressed, as they are the product of the same gene. Molecular mass markers are shown to the left in kDa.

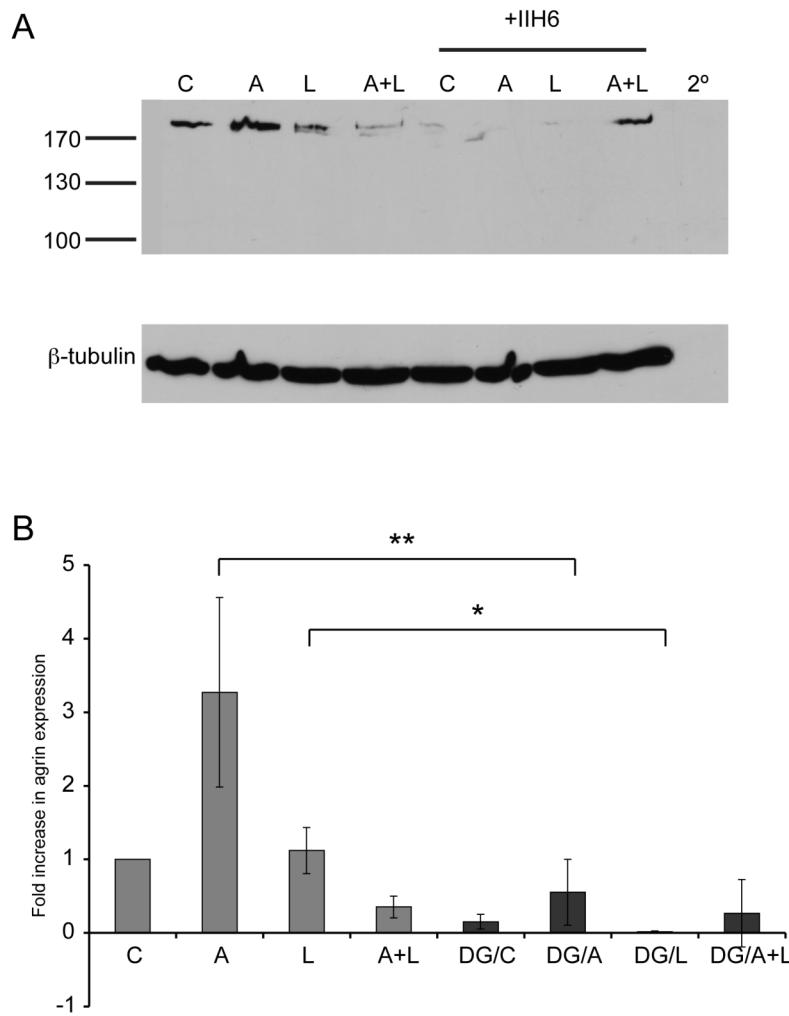


Figure 11. Blocking α -DG reduces the induction of endogenous agrin.

A representative immunoblot for endogenous agrin expression in RAW264.7 cells is shown in panel A. Cells were blocked with 11H6 (DG) at a 1:100 dilution or left unblocked prior to treatment with either agrin (A), LPS (L), agrin and LPS (A+L), or left untreated as a control (C). Endogenous agrin expression levels were quantified by densitometry and are presented as averages (mean \pm SD) relative to the control lane (panel B). Antibody blocked samples reveal an 83% reduction in endogenous agrin in cells treated with agrin **(n=3, p<0.0001 ANOVA/Tukey's HSD p<0.001 and 98% in cells treated with LPS *(n=3, p<0.0001 ANOVA/Fisher's LSD p<0.05).

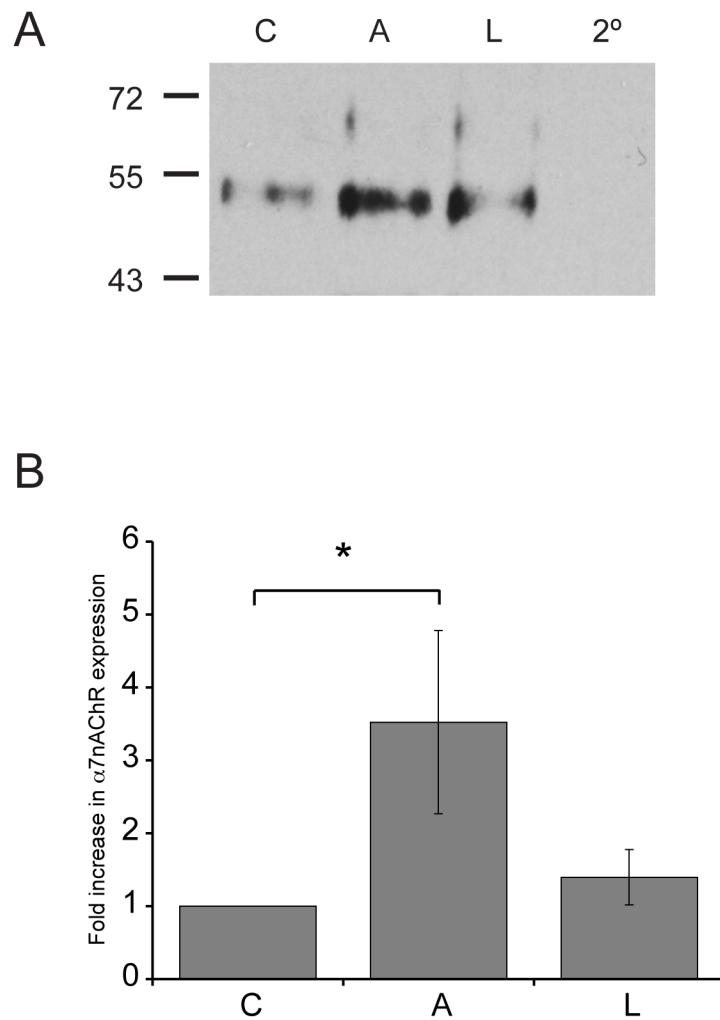


Figure 12. Agrin induces increased expression of $\alpha 7$ nAChRs on macrophages.

RAW264.7 cells were treated with agrin (A), LPS (L), or left untreated (C) for four hours prior to extraction and precipitation with α -BTX-biotin. A representative immunoblot for $\alpha 7$ nAChR expression is shown in panel A. Molecular mass markers are shown on the left in kDa. Densitometry was subsequently utilized for analysis of multiple blots and the values presented in panel B are the means \pm SD of agrin or LPS induction of $\alpha 7$ nAChR compared to control. Agrin treated macrophages show a significant 3.52 ± 1.25 fold increase in $\alpha 7$ nAChR expression when compared to our control values *(n=3, p<0.05, Student's t-test).

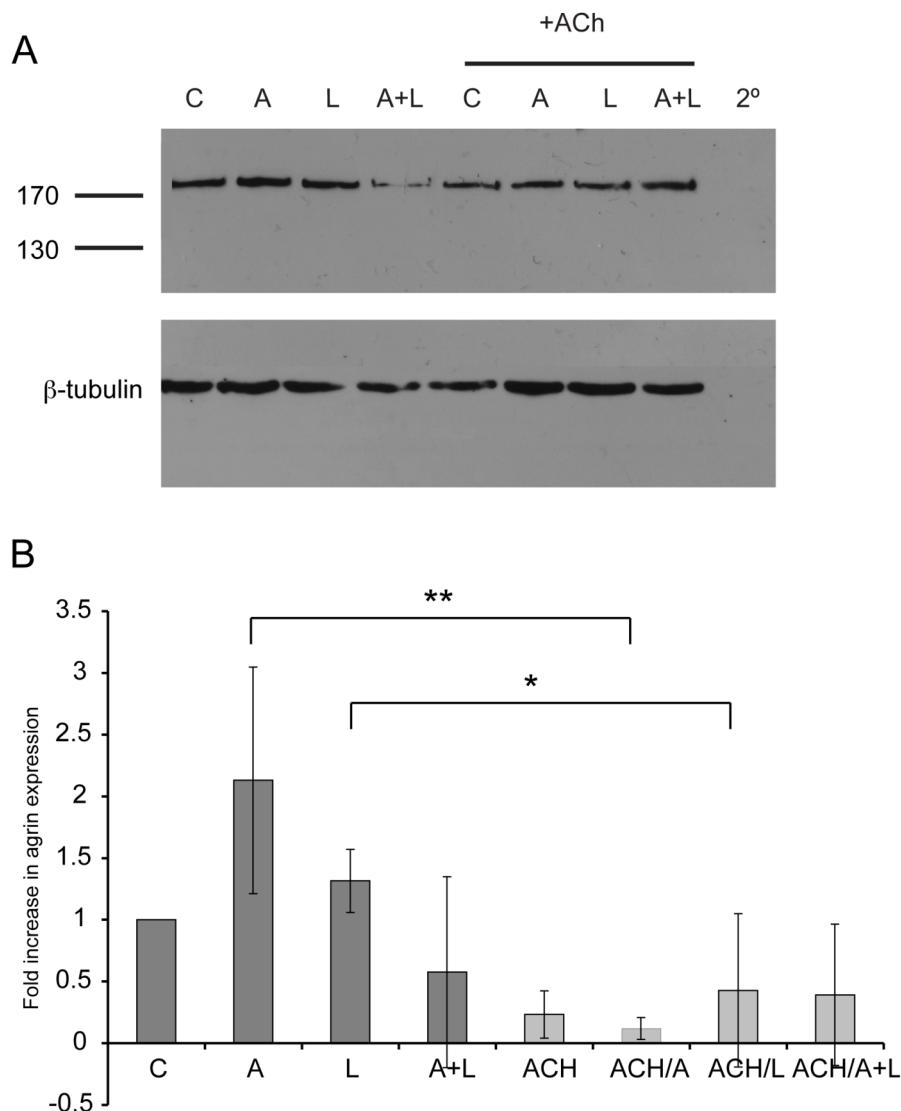


Figure 13. Acetylcholine negates agrin's effects on the induction of endogenous agrin.

RAW264.7 macrophages were treated with agrin (A), LPS (L), agrin and LPS (A+L), or left untreated (C), in the presence or absence of acetylcholine (ACh). A representative western blot for endogenous agrin expression is shown in panel A. Molecular mass markers are displayed in kDa on the left. Densitometric analysis of endogenous agrin expression is represented as averages (mean \pm SD) compared to control (panel B). Acetylcholine pre-treatment reduced agrin expression in agrin treated macrophages by 94% **(n=4, $p<0.001$ ANOVA/Tukey's HSD $p<0.001$) and in LPS treated cells by 67% *(n=4, $p<0.001$ ANOVA/Fisher's LSD $p<0.05$) compared to the control.

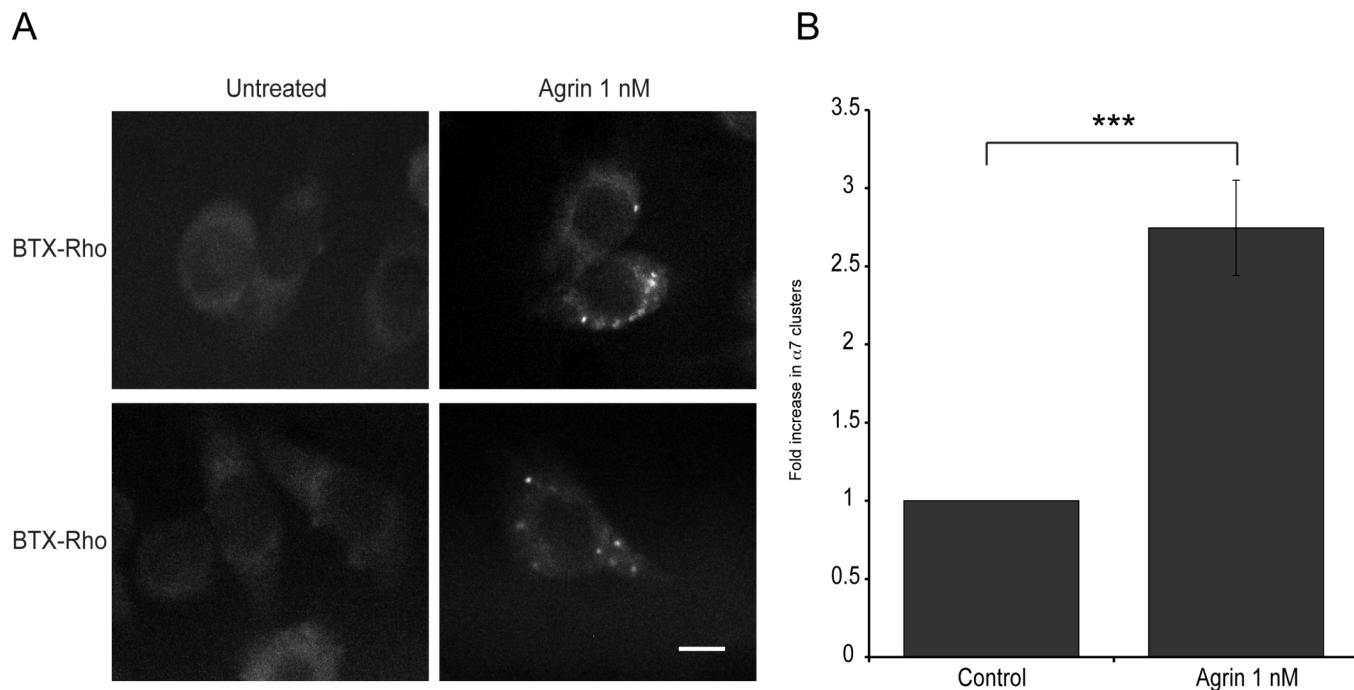


Figure 14. Agrin induces clustering of surface $\alpha 7$ nAChRs on macrophages.

RAW264.7 cells were either treated with agrin (1nM) or left untreated for four hours. Following treatment, cells were incubated with rhodamine-conjugated α -bungarotoxin (BTX-Rho) for one hour, washed then fixed with 4% paraformaldehyde. α 7nAChR aggregates can clearly be seen on the agrin treated cells (above, right, panel A). Scale bar is 10 μ m. In panel B, agrin-induced α 7nAChR clusters were quantified relative to spontaneous clusters observed in control cells and are represented as average # of clusters/cell (means \pm SE). Counts were obtained from at least 5 fields. All AChR clusters were counted, independent of size. Agrin treated macrophages demonstrate a 2.74 ± 0.30 fold increase in α 7nAChR clusters (***($p<0.0001$, Student's *t*-test).

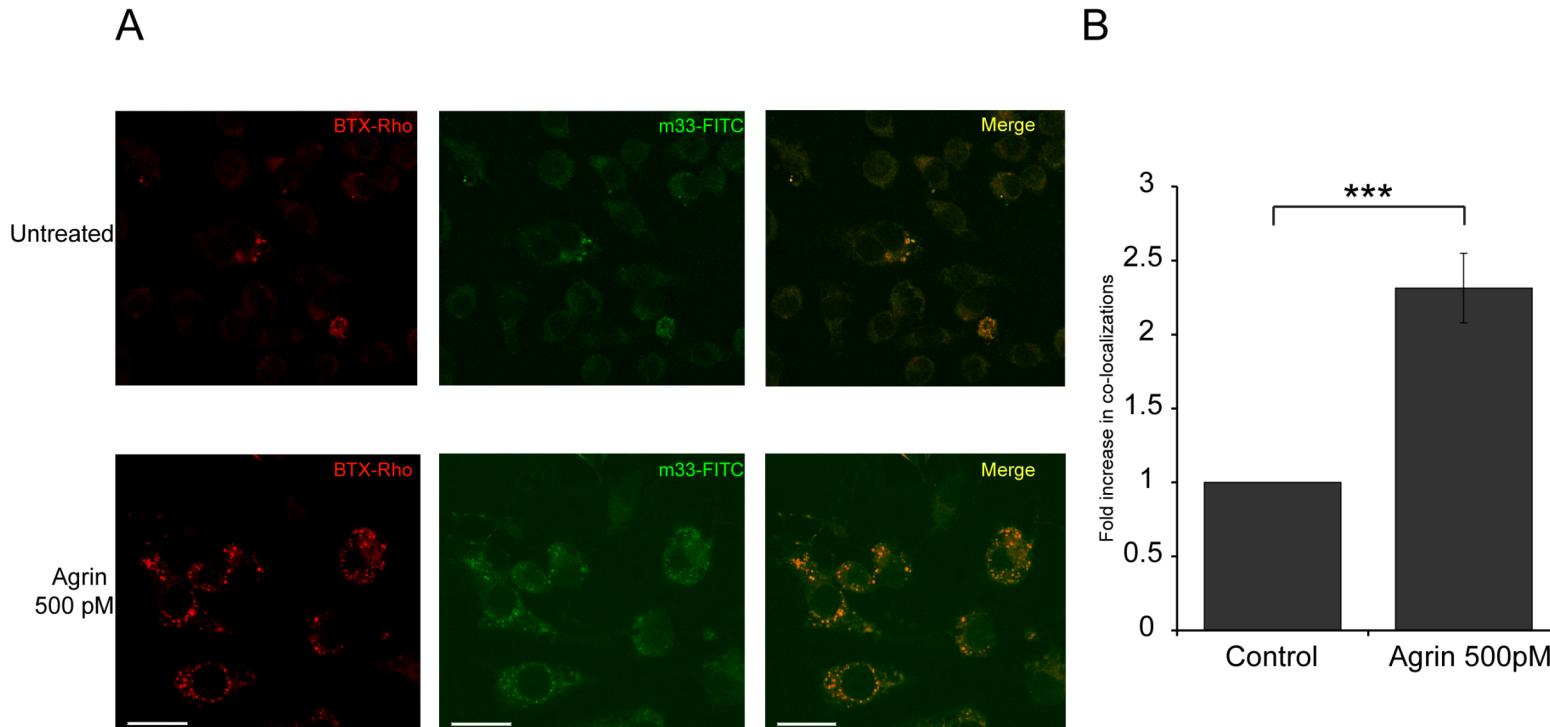


Figure 15. Tm-agrin and α 7nAChRs co-localize on macrophages.

RAW264.7 macrophages were either treated with agrin (500pM) or left untreated for four hours. Following treatment cells were incubated with BTX-Rho (red) to stain for α 7nAChRs for one hour, washed then fixed with 2% paraformaldehyde. Cells were then permeabilized and incubated with monoclonal antibody m33 against agrin, followed by a FITC antibody (green). Fluorescent images were obtained using confocal microscopy and an artificial merge (yellow) was used to show co-localizations (panel A). Increased co-localization is visible in agrin treated cells (bottom, right, panel A). Scale bar is 20 μ m. In panel B, cells showing distinct clusters of co-localization were counted, quantified, and represented as average # of clusters/cell (means \pm SE). Counts were obtained from at least 5 fields. Agrin treated macrophages demonstrate a 2.31 ± 0.24 fold increase in co-localizations ***($p<0.0001$, Student's *t*-test).

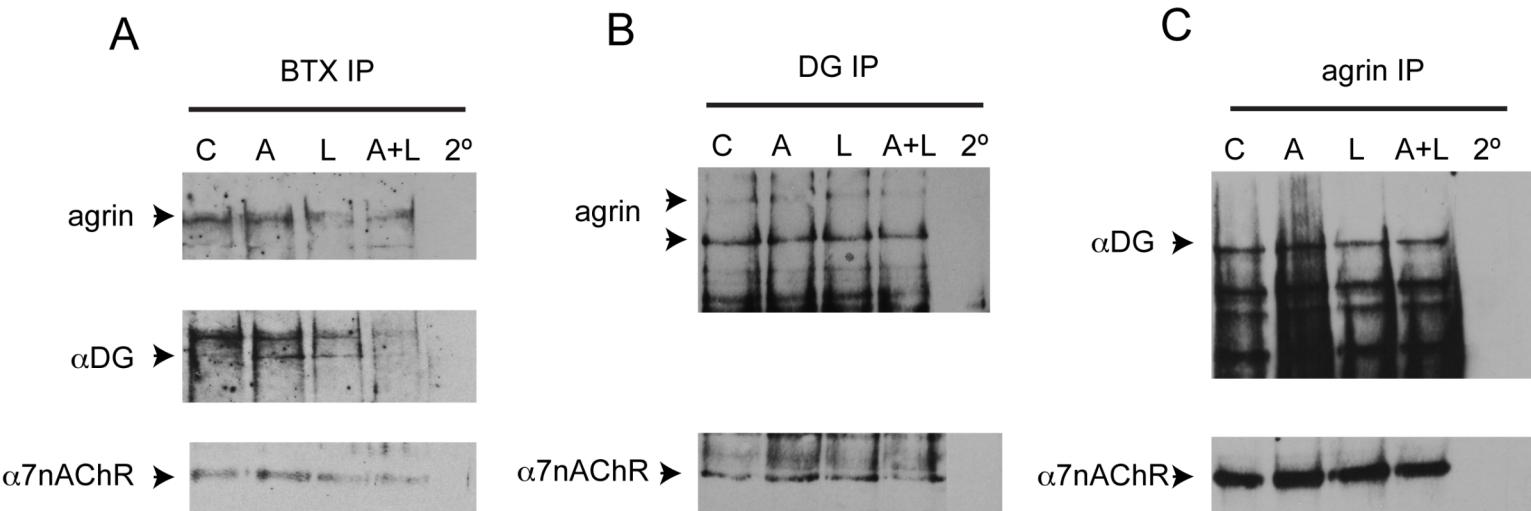


Figure 16. Agrin and α 7nAChRs directly interact with the agrin receptor, α -DG.

Total cell extracts obtained from RAW264.7 cells left untreated (C), treated with agrin (A), LPS (L), or agrin and LPS (A+L) for four hours, were subjected to immunoprecipitation with BTX-biotin and streptavidin-agarose beads (panel A), anti- α -DG antibody and protein-G beads (panel B), or anti-agrin m33 antibody and protein-G beads (panel C). A representative western blot demonstrating co-immunoprecipitations of α 7nAChRs, α -DG and agrin are shown. Arrows indicate proteins that were probed with antibodies against agrin, α -DG, and α 7nAChRs. Immunoblots show that α 7nAChRs co-immunoprecipitate with agrin and α -DG (panel A), α -DG co-immunoprecipitates with agrin and α 7nAChR (panel B), and agrin co-immunoprecipitates with α -DG and α 7nAChR (panel C), in all treatments compared to control.

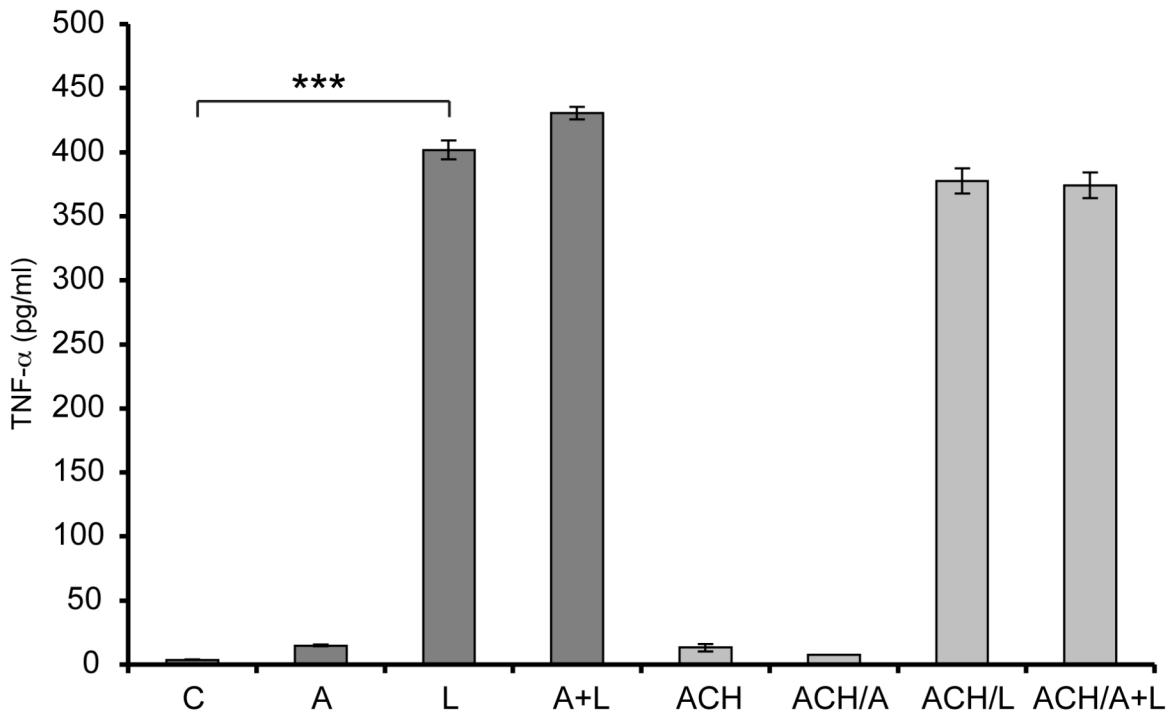


Figure 17. Agrin does not affect TNF- α synthesis in macrophages.

Media from cultures left untreated (C), or treated with agrin (A), LPS (L), or agrin and LPS (A+L), in the presence or absence of ACh (ACH), for four hours, was analyzed for TNF- α concentrations using a commercially available ELISA kit. TNF- α levels induced by agrin (14.82 ± 0.70 pg/ml, n=7) were minimal and comparable to those induced by ACh (13.30 ± 2.86 pg/ml, n=7). These levels are not comparable to the induction of increased TNF- α levels observed in cells challenged with LPS in the absence of ACh ***(401.82 ± 7.39 pg/ml, n=7, $p < 0.0001$ ANOVA/Tukey's HSD $p < 0.0001$).

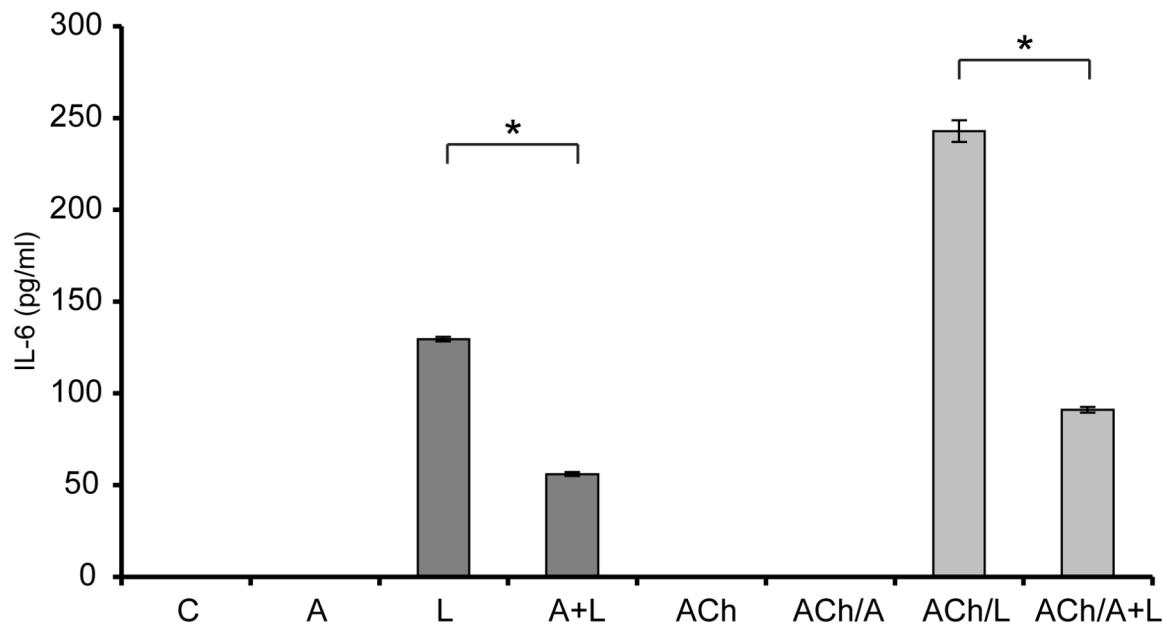


Figure 18. Agrin treatment inhibits IL-6 synthesis in macrophages.

RAW264.7 culture media from untreated (C), agrin treated (A), LPS (L), or agrin and LPS (A+L) treated cells, in the presence or absence of ACh (ACh), was analyzed for IL-6 expression using a commercially available ELISA kit. IL-6 levels decreased by 57% in agrin and LPS treated cells when compared to LPS treated cells alone *(n=3, p<0.0001 ANOVA/Fisher's LSD p<0.05). However, in the presence of ACh, agrin reduced IL-6 levels by 62% in LPS challenged cells compared to LPS in the presence of ACh *(n=3, p<0.0001 ANOVA/Fisher's LSD p<0.05). Thus, agrin in the presence or absence of ACh did reduced IL-6 levels in macrophages.

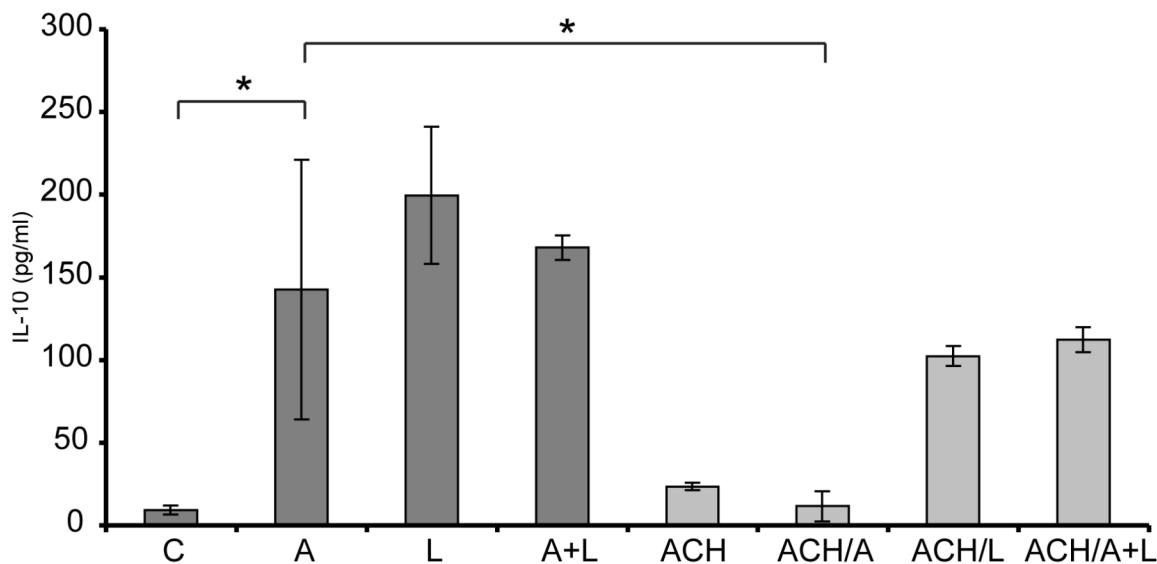


Figure 19. Agrin increases the synthesis of anti-inflammatory IL-10 in macrophages.

RAW264.7 culture media from untreated (C), agrin (A), LPS (L), agrin and LPS (A+L), treated cells in the presence or absence of ACh (ACH), was analyzed for IL-10 concentrations using a commercially available ELISA kit. IL-10 levels were increased 15-fold *(142.67 ± 78.48 pg/ml, n=4, $p < 0.05$ ANOVA/Fisher's LSD $p < 0.05$) in agrin treated macrophages compared to untreated. RAW264.7 cultures pre-treated with ACh and subsequently treated with agrin reduces IL-10 levels by 92% *(11.61 ± 9.05 pg/ml, n=4, $p < 0.05$ ANOVA/Fisher's LSD $p < 0.05$) compared to agrin treated alone.

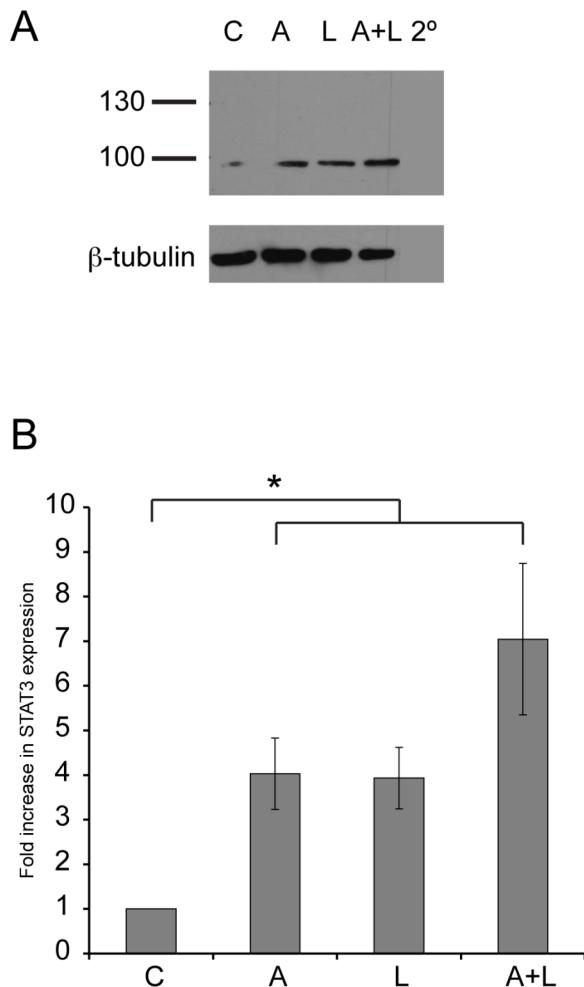


Figure 20. Agrin induces an increase in immunoregulatory STAT3 in macrophages.

A representative western blot for STAT3 in RAW264.7 cells treated with agrin (A), LPS (L), or left untreated (C) for 15 minutes is shown in panel A. Molecular mass markers are shown on the left in kDa. Values obtained by densitometric analysis are represented in panel B represent the means \pm SD relative to control. Agrin treated macrophages show a 4.02 ± 0.80 -fold increase in STAT3 expression *(n=3, p<0.001 ANOVA/Tukey's HSD p<0.05) compared to control. Agrin treated macrophages that have been LPS challenged induced a 7.04 ± 1.70 -fold increase in STAT3 expression *(n=3, p<0.001 ANOVA/Tukey's HSD p<0.05) relative to control.

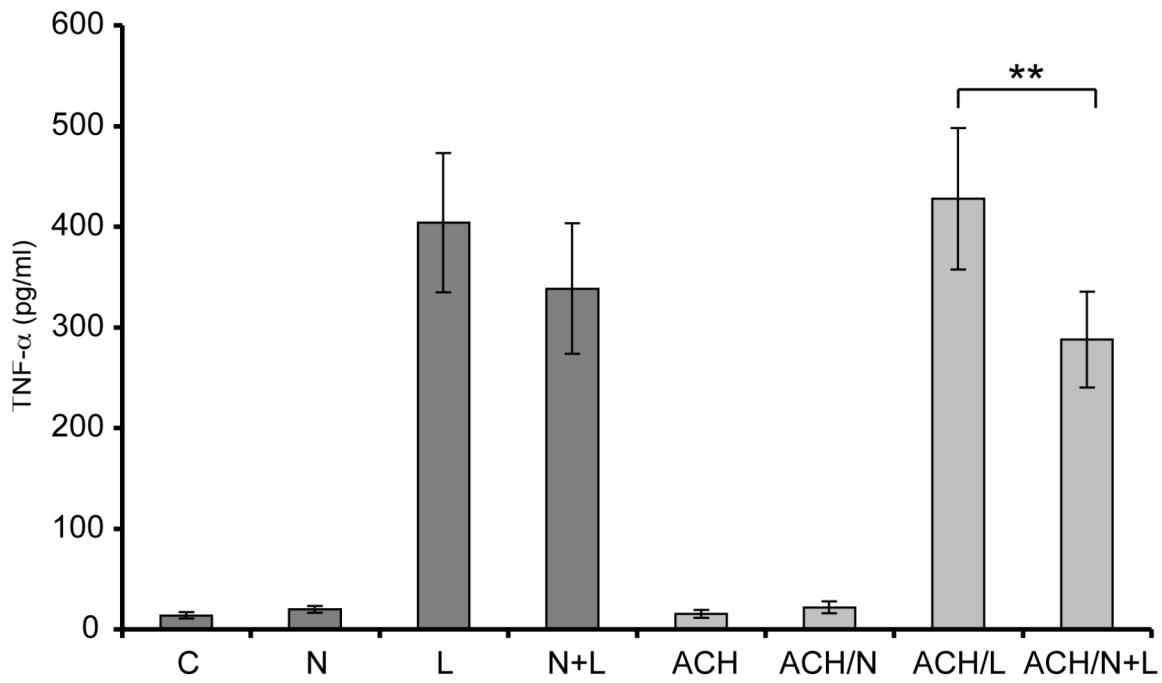


Figure 21. NRG in the presence of ACh reduces the secreted levels of TNF- α in LPS challenged microglia.

Media from BV-2 cultures left untreated (C), or treated with 1nM NRG (N), LPS (L), NRG and LPS (A+L), in the presence or absence of ACh (ACH), for four hours, was analyzed for TNF- α concentrations using a commercially available ELISA kit. TNF- α levels decreased by 16% in LPS challenged microglia treated with NRG compared to LPS treated alone (403.87 ± 69.10 pg/ml, n=7). In the presence of ACh, NRG reduced TNF- α by 33% in microglia challenged with LPS *(n=7, p<0.01, Student's t-test).

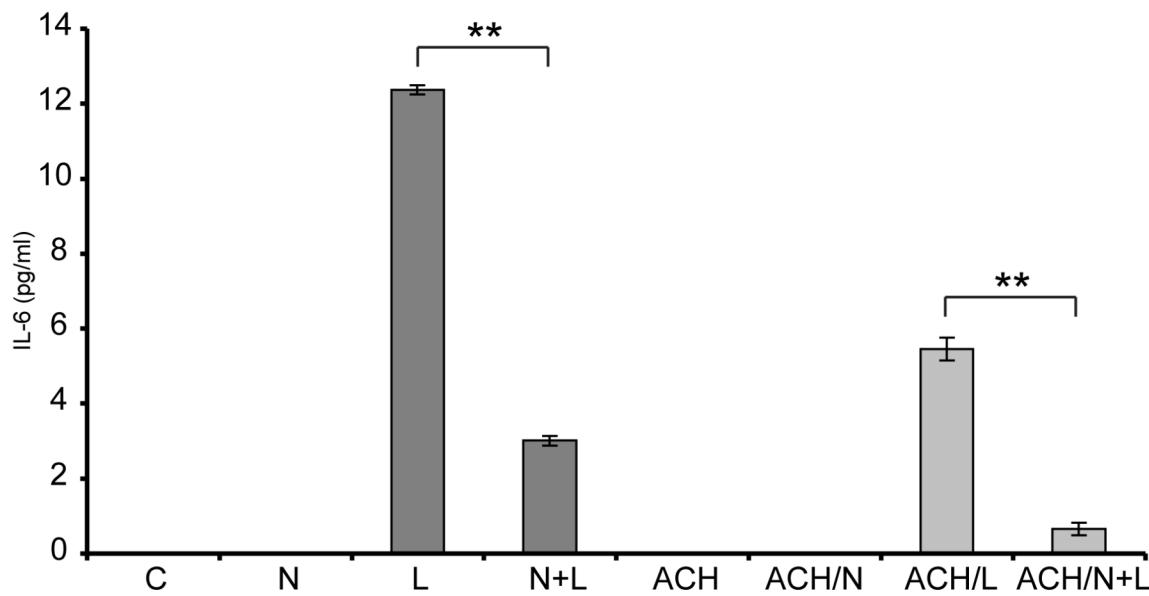


Figure 22. NRG reduces secreted IL-6 levels in the presence or absence of ACh in microglia challenged with LPS.

Media from BV-2 cultures left untreated (C), or treated with NRG (N), LPS (L), NRG and LPS (A+L), in the presence or absence of ACh (ACH), for four hours, was analyzed for IL-6 concentrations using a commercially available ELISA kit. IL-6 levels decreased by 76% in LPS challenged microglia treated with NRG *(n=3, p<0.05, Student's t-test) compared to LPS challenged alone. In the presence of ACh, IL-6 levels decreased by 88% in LPS challenged microglia treated with NRG *(n=3, p<0.05, Student's t-test) compared to LPS challenged microglia in the presence of ACh alone.

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