ROLE OF ASTROGLIAL α7 NICOTINIC ACETYLCOLINE RECEPTORS IN NEUROINFLAMMATION AND OXIDATIVE STRESS

Thesis presented

By

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DEDICATION

I dedicate this dissertation to my best friend and husband, Rishi for his constant support and encouragement during the entire process of this unique journey. I also dedicate this work to my parents and late grandmother for their unconditional love and support, which helped me achieve my dreams.
ACKNOWLEDGMENTS

I would like to acknowledge contributions of some very important people without whom this work would not have been possible. Firstly, I would like to thank both Northeastern University and Biogen for giving me the opportunity to work on my PhD. This has been an amazing learning experience for me. I want to express my deepest appreciation to my two advisors, Dr. Ralph Loring and Dr. Anthone Dunah for their mentorship, guidance and encouragement. A special thank you is due to Dr. Anthone Dunah who in addition to being my advisor is also my supervisor at Biogen, who inspired and encouraged me for career development in the field of research and helped me to balance full time job with my PhD. I also am grateful to the members of my dissertation committee for their valuable feedback which helped tremendously in the completion of my research work. I also want to thank my colleagues at Biogen, whose suggestions always helped me to increase the quality of my research and who always encouraged and supported me throughout this journey. Finally, I would like to extend my gratitude to my friends for always being there for me.
α7 nicotinic acetylcholine receptors (nAChRs) are widely distributed throughout the central nervous system (CNS) and periphery. Within the CNS, these receptors are expressed in neurons and glia cells, and are actively involved in learning, memory and attention. A majority of the studies evaluating the role of α7nAchRs in the CNS have focused on neurons. However, these receptors are also present on astrocytes, which represent 20-40% of the brain cells and are key regulators of neuroinflammation and oxidative stress in several neurodegenerative diseases including Alzheimer’s disease, Parkinson’s disease or amyotrophic lateral sclerosis. Less evidence exists regarding the potential anti-inflammatory properties of these receptors in astrocytes. Therefore, we evaluated the role of α7 nAChR activation in both in vitro and in vivo models of neuroinflammation and aimed to elucidate the molecular mechanism of anti-inflammatory and anti-oxidant properties of these receptors in astrocytes.

We observed that treatment with α7 nAChR agonists, GTS21 and PNU282987, significantly reduced lipopolysaccharide (LPS)-mediated secretion of the inflammatory cytokines in a dose dependent manner in astrocytes and this effect was reversed by pharmacological inhibition of α7 nAChR with the antagonist methyllycaconitine (MLA) and by knocking down α7 nAChR expression with short hairpin RNA suggesting specificity of the response. Further, we assessed the effect of α7 nAChR agonist on activation of NF-κB, which is a transcription factor involved in regulating inflammatory responses. We observed that α7 nAChR activation blocked LPS mediated NF-κB nuclear translocation in astrocytes indicating that the observed anti-inflammatory effect may be mediated through NF-κB pathway. We further tested the antioxidant effect of astroglial α7 nAChR through modulation of nuclear factor erythroid-
derived 2-related factor 2 (Nrf2) pathway, which is a member of the NF-E2 family of basic region leucine-zipper transcription factors and responds to oxidative and electrophilic stress by regulating antioxidant responsive genes. We demonstrated that treatment with α7 nAChR agonists up regulated canonical Nrf2 antioxidant genes and proteins suggesting antioxidant properties of α7 nAchR in astrocytes. Interestingly, α7 nAchR activation in astrocyte cultures from Nrf2 knockout mice not only showed reduction of the anti-oxidant response, but also the anti-inflammatory response in the in vitro inflammation model; highlighting the possibility that cross-talk between the Nrf2 and NF-κB pathways may be responsible for the observed anti-inflammatory properties of α7 nAchR. Using an astrocyte conditioned media approach; we demonstrated reduction in neuronal apoptosis measured by apoptotic marker caspase 3/7 when astrocytes were pre-treated with α7 nAchR agonists. Finally, in an in vivo neuroinflammation model using LPS in NF-κB luciferase reporter mice, we demonstrated reduction with GTS21 treatment in NF-κB activity using whole body imaging and ex vivo brain imaging. We also observed significant reduction in gene expression of pro-inflammatory cytokines and increase in Nrf2 target genes with GTS21 treatment in liver and brain tissues.

In conclusion, our results suggest that activating astroglial α7 nAChRs may have a role in neuroprotection by decreasing inflammation and oxidative stress, and therefore could have therapeutic implication for disease modifying treatments of neurodegenerative diseases.
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<table>
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</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>Aβ</td>
<td>amyloid beta</td>
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<tr>
<td>BBB</td>
<td>Blood–brain barrier</td>
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<tr>
<td>Cat</td>
<td>Catalase</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline acetyltransferase</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-Aminobutyric acid</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GCLC</td>
<td>glutamate-cysteine ligase catalytic subunit</td>
</tr>
<tr>
<td>GCLM</td>
<td>glutamate-cysteine ligase modifier subunit</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>HMGB1</td>
<td>high mobility group protein B1</td>
</tr>
<tr>
<td>HO1</td>
<td>heme oxygenase 1</td>
</tr>
<tr>
<td>IκB</td>
<td>nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha</td>
</tr>
<tr>
<td>IBA1</td>
<td>ionized calcium-binding adapter molecule 1</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin 6</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>IL10</td>
<td>interleukin 10</td>
</tr>
<tr>
<td>IL12</td>
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<tr>
<td>IL13</td>
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<tr>
<td>IL1a</td>
<td>interleukin 1a</td>
</tr>
<tr>
<td>IL1B</td>
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</tr>
<tr>
<td>IL2</td>
<td>interleukin 2</td>
</tr>
<tr>
<td>IL4</td>
<td>interleukin 4</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>Jak2</td>
<td>janus kinase2</td>
</tr>
<tr>
<td>Keap1</td>
<td>Kelch like ECH-associated protein 1</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MPP+</td>
<td>1-methyl-4-phenylpyridinium ion</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>nAChRs</td>
<td>α7 nicotinic acetylcholine receptors</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NO2 –</td>
<td>nitrite</td>
</tr>
<tr>
<td>NQO1</td>
<td>NAD(P)H:quinone oxidoreductase-1</td>
</tr>
<tr>
<td>Nrf2</td>
<td>nuclear factor erythroid-derived 2-related factor 2</td>
</tr>
<tr>
<td>OSGIN1</td>
<td>oxidative stress induced growth inhibitor 1</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>Prdx2</td>
<td>peroxiredoxin</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SOCS3</td>
<td>suppressor of cytokine signalling 3</td>
</tr>
<tr>
<td>SRXN1</td>
<td>sulfiredoxin-1</td>
</tr>
<tr>
<td>STAT3</td>
<td>signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>Trx</td>
<td>thioredoxin</td>
</tr>
<tr>
<td>TXNRD1</td>
<td>thioredoxin reductase 1</td>
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INTRODUCTION

Statement of the problem

Neuroinflammation and oxidative stress are hallmarks of several neurodegenerative diseases.\textsuperscript{1} $\alpha_7$nAchR are reported to have anti-inflammatory properties in the peripheral nervous system,\textsuperscript{2} and are highly expressed in the brain and reported to be neuroprotective.\textsuperscript{3-5} A majority of the studies evaluating the role of $\alpha_7$nAchRs in the central nervous system have focused on neurons. However, these receptors are also present on astrocytes, which is a key regulator of neuroinflammation and oxidative stress in several neurodegenerative diseases. A few studies have been conducted to demonstrate anti-inflammatory properties of $\alpha_7$nAchRs in glial cells.\textsuperscript{6,7} However, the molecular mechanism of the observed anti-inflammatory response remains unclear. Therefore, in this dissertation, we evaluated the anti-inflammatory and anti-oxidant properties of astroglial $\alpha_7$nAChR activation and aimed to demonstrate the molecular mechanism for these effects. We hypothesized that the NF-κB pathway, the Nrf2 pathway, and the interplay between the two mediate this effect. Specifically, we evaluated the following Aims,

\textbf{Aim 1:} To develop, optimize and validate a protocol for culturing astrocytes from rodent brain and evaluate anti-inflammatory properties of astroglial $\alpha_7$nAchR

\textbf{Aim 2:} To study the effect of $\alpha_7$nAchR activation on Nrf2 signaling in astrocytes

\textbf{Aim 3:} To investigate the effect of astroglial $\alpha_7$nAchR activation on neuronal survival

\textbf{Aim 4:} To study the effect of $\alpha_7$nAchR activation in an \textit{in vivo} neuroinflammation model
Review of literature

I. Astrocytes

Astrocytes are the most abundant cell type in the brain, representing 20 to 40% of all brain cells.\textsuperscript{8} The density of astrocytes in brain varies by region. In cerebral cortex they are more commonly found as compared with neurons, but in cerebellum they are less frequent. Astrocytes are originated postnatally during gliogenesis in rodent brains.\textsuperscript{9} The number of astrocytes increases by many folds in the rodent brain during first few weeks of postnatal development.\textsuperscript{10} Astrocytes have diverse functions in the brain. One of their main roles is maintenance of synaptic function and plasticity by balancing homeostasis of neurotransmitters and ions.\textsuperscript{11} Astrocytes are fundamental components of the ‘tripartite’ synapse, which consists of pre-synaptic membrane, post-synaptic membrane and astrocytes which surrounds the peri-synaptic area. They modulate synaptic strength and efficacy at excitatory and inhibitory synapses through expression of neurotransmitter receptors and release of a variety of neurotransmitters including glutamate, GABA, and D-serine via Ca\textsuperscript{2+}-dependent exocytosis.\textsuperscript{11} Astrocytes also play a critical role in ion homeostasis. They contain glutamate receptors, which induces an increase intracellular Ca\textsuperscript{2+} concentration in response to glutamate release.\textsuperscript{12} They regulate extracellular K\textsuperscript{+} level by uptake of this ion through transporters or channels when the extracellular concentration is increased. This K\textsuperscript{+} is transferred to adjacent astrocytes via gap junctions by a process called spatial buffering.\textsuperscript{13} This process prevents K\textsuperscript{+} concentration from reaching toxic levels.

Astrocytes also play a role in blood flow and neuronal energy metabolism. They provide metabolic support to neurons through the astrocyte-neuron lactate shuttle in which lactate is released by astrocytes. This lactate is converted to pyruvate, which in turn is taken up into
neurons for their energy metabolism. Another crucial function of astrocytes is formation of the blood brain barrier through their end-feet contacting capillary endothelial cells. Astrocytes play a critical role in the brain’s defense system. In the adaptive immune system, they can act as phagocytic cells and possess antigen presenting capabilities. Importantly, they also act as mediators of the innate immune system by producing a wide range of chemokines and cytokines in response to stimulation by toxic and traumatic insults. This complex process, termed as reactive astrogliosis, involves morphological and functional changes in astrocytes. These changes include hypertrophy, upregulation of intermediate filaments, such as glial fibrillary acidic protein (GFAP), and increased proliferation.

Figure 1- Astrocyte functions in healthy CNS and change in morphology in response to injury

II. Astrocytes mediated neuroinflammation

Chemokines and cytokines produced during reactive astrogliosis play an important role in recruitment of leukocytes to inflammatory sites. Therefore, in the event of reactivation of astrocytes of the BBB, the released chemokines attract leukocytes across the BBB to initiate neuroinflammation in the CNS. Reactive astrogliosis is observed during a variety of conditions including brain or spinal cord infections, injuries to the brain, spinal cord and retina, epilepsy, stroke, some brain tumors, and neurodegenerative diseases, e.g., Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis. Reactive astrogliosis serves as a physiological response to the CNS insult and minimize and repair the initial damage in the early phases of these conditions. However, in situations of prolonged brain insults, sustained inflammatory responses driven by positive feedback loops can lead to chronic neuroinflammation and provide detrimental signals, which eventually compromise astroglial and neuronal functions. Since in neurodegenerative diseases, such prolong brain insults are commonly observed, astrogliosis leads to chronic inflammation and plays a critical role in the pathophysiology of these diseases. Below we describe various neurodegenerative diseases with known astrocytic contribution.

1. Alzheimer’s disease (AD): AD is the most prevalent neurodegenerative disease in the US and is characterized by cognitive impairment. This impairment results from neuronal dysfunction and death. Pathologically, amyloid plaques, which contains the protein amyloid beta (Aβ) and neurofibrillary tangles containing hyperphosphorylated tau protein, are hallmarks of AD. A recent study by Webster et al. showed that in patients with early AD, there was significant astrogliosis through increased phosphorylation of
astrocytic extracellular signal-regulated kinase (ERK) in the white matter that was correlated with the severity of cognitive decline and neuropathological stage. Evidence from a multitracer PET imaging study conducted in AD patients with mild cognitive impairment suggested that reactive astrocytes are detected at early stages of the disease.\textsuperscript{21} Similar results from animal models of AD have also been documented suggesting presence of reactive astrocytes before deposition of amyloid-beta.\textsuperscript{22} Another study documented presence of reactive astrocytes surrounding the amyloid plaques in mouse AD model.\textsuperscript{23} Aβ induced astrogliosis initiates a neuroinflammatory cascade; in which the proinflammatory cytokines and chemokines, including interleukins (IL)-1, IL-6, transforming growth factor (TGF) and tumor-necrosis factor (TNF), are released.\textsuperscript{18} These cytokines and chemokines lead to neuroinflammation and play a predominant role in early AD pathogenesis.

2. \textbf{Parkinson’s disease (PD):} PD is the second most common neurodegenerative disease and is characterized by tremor, bradykinesia, rigidity and loss of postural coordination. Pathological hallmarks of the disease include loss of dopaminergic neurons in substantia nigra and appearance of α-synuclein and Lewy bodies in the cytoplasm of remaining neurons. In PD, astrocytes do not undergo reactivation, but instead play a role in the initiation and progression of the disease by promoting accumulation of α-synuclein in neurons.\textsuperscript{24} Experimental evidence suggests that this accumulation initiates the non-cell autonomous killing of neurons through microglial signaling.\textsuperscript{25}
3. **Amyotrophic Lateral Sclerosis (ALS):** ALS is another neurodegenerative disease characterized by rapid degeneration of motor neurons. ALS progresses very rapidly to cause paralysis and death within a few years. Normally, astrocytes closely interact with neurons and provide an optimal environment for neuron growth and functioning. In ALS, reactive astrocytes are known to surround degenerating motor neurons and are thought to influence motor neuron fate, potentially through a selective impairment in glial glutamate transport leading to excitotoxic levels of glutamate and through upregulation of iNOS expression leading to oxidative and nitrative stress.\(^{26}\) The potential therapeutic value of astrocytes as a target is further highlighted by observations that riluzole, a treatment that delay the onset or improve survival in ALS patients, decrease reactive astrogliosis.\(^{27}\)

**III. \(\alpha7\) nicotinic acetylcholine receptors (nAChRs)**

nAChRs are found abundantly in both periphery and brain. In periphery, they are located in neuromuscular junctions of somatic muscles, while in brain they are found in neuronal and non-neuronal cells. Neuronal nAChR are cation selective ligand gated ion channels are permeable to \(\text{Na}^+\), \(\text{K}^+\), and \(\text{Ca}^{2+}\). They comprise of pentameric complexes of alpha and beta subunits. In total, nine alpha and four beta neuronal subunits have been identified. Two adjacent cysteine residues contained on alpha subunits act as agonist binding site and are responsible for binding of acetylcholine.\(^{28}\)
Combination of alpha and beta subunits determines the sensitivity to agonists and antagonists. Some known agonists for these receptors include endogenous neurotransmitter acetylcholine and
exogenous ligand nicotine, and antagonists include curare, α-bungarotoxin, methyllycaconitine (MLA) or mecamylamine.\textsuperscript{29}

An important nAChR subtype in the brain is α7; which is structurally unique from other nAChRs because it is only composed of five alpha subunits (\textbf{Figure 2}). α7 subtype is also functionally distinct from other subtypes with its characteristic low sensitivity to nicotine and faster desensitization kinetics.\textsuperscript{30} These are expressed throughout the brain regions including cortex, hippocampus, substantia nigra, cerebellum, and amygdala. One of the major functions of α7 nAChRs in CNS is regulation of neurotransmitter release, which is achieved by pre-synaptically located receptors. It promotes ACh release, which in turn plays a role in attention, learning, and memory. Post-synaptic α7 nAChRs are known to regulate depolarization of neurons and thereby modulating cell-excitability.

**IV. α7 nAChR implications in neurodegenerative disorders**

α7 nAChRs have been of great interest as potential therapeutic targets in various neurodegenerative diseases; owing largely to past observations of their neuroprotective effects. Observations from cell lines and primary neuron cultures indicate that agonists of these receptors, including galantamine (an allosteric modulator), PNU-282987, TC-1698, and GTS21 (3-[2,4-dimethoxybenzylidene]anabaseine), provide neuroprotection against toxicity induced by various insults such as amyloid-beta, glutamate, okadaic acid, and ethanol.\textsuperscript{31-34} Conversely, prior studies also document a blockage of neuroprotection by α7 nAChR antagonists such as methyllycaconitine (MLA) or α-bungarotoxin against toxic insults including amyloid-beta, glutamate and NMDA as well as oxygen–glucose deprivation in cell lines and primary neuronal cultures.\textsuperscript{35-37} Further, experimental evidence from α7 nAChR knock-out mice also supports this
observation; where nicotine did not exhibit any neuroprotective effect against oxygen and glucose deprivation in transgenic mice with deleted α7 nAChR. In sum, observations in experiments conducted using receptor targeted agents and genetically modified animals strongly suggest a neuroprotective role of α7 nAChR against numerous toxic insults. Below we describe various neurodegenerative diseases where α7 nAChRs could be of potential therapeutic value.

1. **Alzheimer’s Disease (AD):** α7 nAChRs are seen as an extremely relevant target for AD due to several important reasons. First, these receptors are highly expressed in hippocampus; which is a region particularly affected in AD. Second, observations suggest that activation of α7 nAChRs enhances long-term potentiation resulting in improved cognition; which is a major challenge in AD. Finally, α7 nAChRs are known to interact with amyloid-beta; which is the most widely accepted hallmark of AD. In human sporadic AD brains, α7 nAChRs have been shown to be present in amyloid plaques bound to amyloid-beta with very high affinity. Indeed, several pre-clinical studies of selective agonists of α7 nAChRs have been successful in restoring cognition in mice models; clearly indicating the importance of these receptors as a viable target in AD. A partial agonist of α7 nAChRs, EVP 6124, is currently undergoing clinical trials as a potential disease modifying therapy for AD.

2. **Parkinson’s disease (PD):** Experimental evidence suggests that α7 nAChRs can also be an important target for PD. In parkinsonian animal models, α7 nAChR agonists have been shown to protect against nigrostriatal damage induced by 6-hydroxydopamine in rats as well as MPTP in mice. Researchers have also successfully blocked the effect of
niogrostriatal protection with α7 nAChR antagonists; further providing evidence for a role of α7 nAChRs in protection against nigrostriatal damage. Additionally, numerous studies have observed modulation of neuritic growth via α7 nAChR mediated alteration in intracellular calcium suggesting a trophic role of α7 nAChRs in CNS.\textsuperscript{47,48} Taken together with a protective effect against nigrostriatal damage; these findings suggest that α7 nAChRs may have disease modifying potential in PD.

3. Amyotrophic lateral sclerosis (ALS): Limited evidence suggests that α7 nAChRs may be involved in the pathogenesis of ALS. Using an \textit{in vitro} ALS model from rat spinal cord cultures, Nakamizo et al.\textsuperscript{36} demonstrated that nicotine rescued glutamate-induced motor neuronal death. Further, this neuroprotection was inhibited by α-bungarotoxin; suggesting mediation of this effect through α7 nAChRs. Thus, α7 nAChRs may also be a potential target for development of therapeutics for ALS.

V. Mechanism of α7 nAChR anti-inflammatory response

\textit{The cholinergic anti-inflammatory reflex}

Upon infection and inflammatory response by bacterial components or intracellular mediators such as high mobility group protein B1 (HMGB1), macrophages are activated and produce pro-inflammatory cytokines. This triggers signaling of afferent vagus nerve; which will transmit information to the brain. As a result, efferent vagus nerve is activated, which will transmit signal through celiac ganglion into splenic nerve and result in generation of nor-epinephrine release. Nor-epinephrine will cause release of ACh by ChAT+ T-cells. This ACh interacts with α7
nAChRs present on splenic macrophages and blocks the secretion of pro-inflammatory cytokines resulting in suppression of the inflammatory response (Figure 3).\textsuperscript{49} This observation, termed the cholinergic anti-inflammatory reflex, suggests that peripheral α7 nAChRs play an important role in regulation of inflammation.

**Figure 3- The cholinergic anti-inflammatory response**

(From Andersson and Tracey, Journal of Experimental Medicine, 2012)

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**Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway**

NF-κB is a transcription factor negatively implicated in cell survival based on its role in inflammatory response. Normally, NF-κB is restricted in the cytoplasm due to binding with IκB. Nuclear translocation of NF-κB requires phosphorylation and ubiquitination of the IκB. Upon nuclear translocation, it regulates the transcription of pro-inflammatory cytokines such as TNF and IL-6.\textsuperscript{50,51} In non-neuronal cells such as monocytes and macrophages, anti-inflammatory
property of α7 nAChRs is reported to be mediated through inhibition of NF-κB.\textsuperscript{50,52} More recently, it has been demonstrated that activation of α7 nAChRs in glial cells leads to blocking of NF-κB pathway and consequent reduction in neuroinflammation.\textsuperscript{53}

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**Figure 4- Subcellular mechanism of nicotinic anti-inflammatory pathway**

(From de Jonge and Ulloa, British Journal of Pharmacology, 2007)

The putative mechanism of NF-κB inhibition with α7 nAChR activation involves janus kinase2 / signal transducer and activator of transcription 3, (Jak2/STAT3) signaling (Figure 4). α7 nAChR activation is thought to result in the recruitment and phosphorylation of tyrosine kinase Jak2 and
subsequent activation of the transcription factor STAT3.\textsuperscript{54,55} STAT3 negatively regulates NF-κB binding to the DNA and facilitates activation of suppressor of cytokine signalling 3 (SOCS3), which in turn contributes to the anti-inflammatory response.\textsuperscript{56,56} In research studies using cells that express STAT3 mutated in the phosphorylation or the DNA binding domain, nicotine failed to produce anti-inflammatory effects suggesting that activation of STAT3 signaling is vital for anti-inflammatory properties of nicotine.\textsuperscript{54} STAT3 is known to be an anti-inflammatory transcription factor,\textsuperscript{57,58} that demonstrate its anti-inflammatory effects indirectly and not by direct inhibition of pro-inflammatory cytokines.\textsuperscript{59}

\textit{Nuclear factor E2-related factor 2 pathway}

Levels of reactive oxygen species (ROS) are tightly regulated in CNS. This is important because brain cells are extremely sensitive to oxidative stress due to high amount of polyunsaturated fatty acids and trace metal ions. This causes them to have increased risk of formation of reactive hydroxyl radicals with hydrogen peroxide and also susceptible to lipid peroxidation. Therefore under physiological conditions, cells maintain redox homoeostasis by exerting its endogenous antioxidant response. Under pathological condition such as neurodegenerative diseases, exaggerated levels of ROS are produced. This will exhaust the endogenous cellular antioxidant defense mechanism, resulting in significant oxidative stress and thereby causing neuronal loss.\textsuperscript{60} Oxidative stress is a common pathological insult contributing to the development of various neurodegenerative diseases. In AD, amyloid β induces oxidative stress by alteration of mitochondrial membrane potential, which results in mitochondrial dysfunction.\textsuperscript{61} Further, binding of amyloid β to copper ions results in generation of free radicals and cytotoxicity.\textsuperscript{62} In PD, loss of functionality of the mitochondrial protein parkin results in mitochondrial dysfunction.
by oxidative stress.\textsuperscript{63} Mutation in another protein DJ-1, which normally protects against oxidative stress by stabilizing Nrf2, results in loss of its protective functions and increase the susceptibility of dopaminergic neurons to oxidative stress.\textsuperscript{64} Similarly, in Huntington’s disease (HD), mutated and aggregated forms of huntingtin has been shown to increase cytotoxicity through increase in ROS production.\textsuperscript{65} Further, mutated SOD1 protein genes are associated with impaired mitochondrial functioning and increased oxidative stress in ALS.\textsuperscript{66} Another neurodegenerative disease in which oxidative stress plays a significant role is multiple sclerosis (MS). High levels of ROS and RNS produced by macrophages and microglia promote leukocyte migration and injury to oligodendrocytes, which is a pathological hallmark of MS.\textsuperscript{67-69}

Nuclear factor E2-related factor 2 (Nrf2) is a transcription factor known to maintain redox balance by regulating transcription of several endogenous antioxidant enzymes (Figure 5). In physiological conditions, the levels of Nrf2 are maintained by constitutive synthesis and degradation with help from the repressor protein Kelch-like ECH-associated protein-1 (Keap1). Keap1 binds to an E3 ubiquitin ligase complex (Rbx-1) via an adaptor protein cullin-3 and ultimately results in Nrf2 ubiquitination and proteasomal degradation.\textsuperscript{70} Three cysteine residues on Keap1 are critical for ubiquitination of Nrf2 under normal condition.\textsuperscript{71} However, during oxidative stress, electrophiles and ROS interact with the cysteine residues of Keap1 and changes its conformation. This conformational change of Keap1 causes release of Nrf2 preventing its proteosomal degradation.\textsuperscript{72} Upon dissociation from Keap1, Nrf2 translocates from cytoplasm to nucleus and binds to the promoter of antioxidant response element (ARE) also known as electrophile response element (EpREs).\textsuperscript{72} Ultimately, this results in transcription of several genes including, heme oxygenase (HO1), glutamate-cysteine ligase modifier subunit (GCLM) and catalytic subunit (GCLC), thioredoxin (Trx) and NAD(P)H:quinone oxidoreductase-1 (NQO1)
involved in phase II detoxification and antioxidant response thereby maintaining redox homeostasis.\(^{73}\)

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**Figure 5- Nrf2 signaling pathway**

(From Buelna-Chontal and Zazueta, Cellular Signaling, 2013)

The process of Nrf2 nuclear translocation and activation is affected by several kinases via specific phosphorylation sites. Atypical PKC iota (aPKCi) phosphorylates Nrf2 at Ser40 causing its release from Keap1 and nuclear translocation.\(^ {74}\) Other kinases that also found to be associated with phosphorylation and activation of Nrf2 signaling include phosphatidylinositol-3-kinases (PI3K),\(^ {75}\) casein kinase-2 (CK2),\(^ {76}\) c-Jun N- terminal kinase (JNK) and extracellular regulated kinase (ERK).\(^ {77}\) Studies have suggested that there are some kinases that negatively regulate Nrf2.
Phosphorylation of Nrf2 by glycogen synthase kinase 3ß increases the degradation of Nrf2 independent from Keap1. Further, NF-kB subunit p65/RelA increases nuclear transport of Keap1, which binds to Nrf2 resulting in its degradation and functional inactivation.

Increased expression of Nrf2 downstream genes, HO1 and NQO1, was observed in the hippocampus and cortex of AD patients compared with healthy controls. However, in postmortem brains of AD patients, Nrf2 was reported to be localized in cytoplasm of hippocampal neurons. Taken together, these observations suggest that in early phases of AD, Nrf2 pathway may try to combat the oxidative stress, but the chronic nature of oxidative stress in AD may render this pathway dysfunctional as evidenced by inability of Nrf2 to translocate in the nucleus. In APP/PS1 transgenic mouse model of AD, reduced Nrf2 levels and inactivation of Nrf2 pathway has been demonstrated. In \textit{in vitro} model of AD, activation of Nrf2 has shown to reduce neurotoxicity against A\beta. Also in transgenic AD animal models, activators of Nrf2 have shown to improve cognitive functioning.

In post-mortem brains of PD pateints, Nrf2 has shown to be translocated into the nucleus but there is insufficient production of anti-oxidant genes suggesting reduced functionality of the Nrf2 pathway. Increased PD pathology observed in Nrf2 knockout mouse models subjected to neurotoxins such as 6-hydroxydopamine (6-OHDA) and MPTP and activation of Nrf2 is shown to be protective. Further, overexpression of Nrf2 in astrocytes has shown to lead to rescue the effects of these toxins.

Major dysfunctions in the Nrf2 pathway have also been noted in ALS. In motor neurons expressing SOD1 mutations, a reduced level of Nrf2 related genes is reported in postmortem ALS patients’ spinal cord. Additionally, a higher level of Keap1 mRNA is reported in the motor cortex of ALS patients. In transgenic mouse model of ALS with overexpression of
mutant SOD1, activation of Nrf2 in astrocytes results in decreased motor neuron death and increased survival of the mice.

Autoimmune encephalomyelitis (EAE) model is a well-known animal model of MS. Induction of EAE in Nrf2 knock-out mice resulted in higher disease severity, higher lesion volume, and increase in infiltrating immune cells.\(^\text{90}\) Also, low levels of Nrf2 were observed in oligodendrocytes at the lesion site and therefore, increasing the susceptibility of these cells to oxidative stress.\(^\text{91}\) One of the disease modifying therapies approved for relapsing remitting MS, dimethyl fumarate, converts to its active metabolite monomethyl fumarate, which is a potent inducer of Nrf2 pathway.\(^\text{92,93}\)

Recent evidence from rat organotypic hippocampal slice culture suggests that \(\alpha7\) nAChR agonists induce hemeoxygenase 1 via Nrf2 pathway in brain ischemic model, which provides neuroprotection.\(^\text{7}\) Further Parada et al.\(^\text{94}\) demonstrated that \(\alpha7\) nicotinic receptor agonist treatment results in inhibition of ROS production in microglia through activation of Nrf2 pathway. Taken together, these studies suggest the involvement of Nrf2 pathway in \(\alpha7\) nicotinic receptor mediated neuroprotection.

**Cross-talk between the NF-\(\kappa\)B and Nrf2 pathways**

In addition to its critical role against oxidative stress, recent observations have implicated Nrf2 as an important player in regulation of inflammatory responses as well. A neurodegenerative phenotype is reported in Nrf2 deficient mice. Innamorato et al.\(^\text{86}\) observed an increased LPS-induced neuroinflammation response in Nrf2 deficient animals compared with the wild-type littermates; leading them to conclude that Nrf2 may be an important regulator of neuroinflammation. The heightened neuroinflammation in the absence of Nrf2 may be explained in part by the interplay between the Nrf2 and NF-\(\kappa\)B pathways. Keap1, which is a negative
regulator protein of Nrf2, is also identified as a binding partner of the p65 subunit of NF-κB. Therefore, upon activation of NF-κB pathway, the NF-κB p65 subunit promotes nuclear translocation of Keap1 from cytoplasm. Increased concentration of Keap1 in the nucleus results in functional inactivation of Nrf2. These observations suggest that activation of NF-κB initiates a cascade that eventually leads to inactivation of Nrf2 and results in a potent inflammatory response. As described in previous sections, α7 nAChR agonists can potentially inhibit NF-κB pathway and activate Nrf2 pathway. Therefore, activation of α7 nAChR is a promising approach to prevent neuronal cell damage owing to intracellular inflammatory responses.

VI. Significance of the proposed research

The primary objective of this dissertation is to evaluate the anti-inflammatory and anti-oxidant effects of astroglial α7 nAChRs activation. Astrocytes represent 20 to 40% of all brain cells and α7 nAChRs are expressed abundantly on cortical and hippocampal astrocytes. Therefore, targeting these receptors for their neuroprotective properties may be important in guiding development of disease modifying treatments for various neurodegenerative diseases. Some preliminary data point towards potential anti-inflammatory effects mediated through α7 nAChRs expressed in astrocytes. Using in vivo mouse model of PD, Liu et al. demonstrated that nicotine administration corrected MPTP-induced behavioral symptoms and these effects were blocked by an α7-nAChR-selective antagonist (MLA). In vitro, 1-methyl-4-phenylpyridinium ion (MPP+) or LPS-induced activation of astrocytes was suppressed by nicotine pre-treatment and MLA reversed these effects. Thus, these data suggest that nicotine-induced anti-inflammatory response occurs via α7-nAChR-mediated inhibition of reactive astrocytes. However, the molecular mechanism of the observed anti-inflammatory response
remains unclear. Therefore, in this dissertation, we propose to evaluate the anti-inflammatory and anti-oxidant properties of astroglial α7-nAChR activation and uncover the molecular mechanism for these effects. We hypothesized that the NF-κB pathway, the Nrf2 pathway, and the crosstalk between the two mediate these effects.
MATERIAL AND METHODS

I. Astrocytes isolation

Astrocytes were purified from cortices of postnatal day 2 C57BL/6 mouse pups using a shaking method described by McCarthy. Cortical tissues were first dissected followed by removal of meninges. The tissues were then briefly centrifuged and washed with cold HBSS. Tissue dissociation was performed by adding papain from Neural Tissue Dissociation Kit (Papain) by Miltenyi Biotec Inc. Tissues were mixed with papain solution using gentle MACs dissociator followed by incubation for 15 min at 37°C. Tissues were further incubated with DNase for 30 seconds and mixed until well dissociated. After dissociation, cells were passed through 40μM sterile filter and centrifuged at 2000 rpm for 10 min. Later the cells were resuspended into DMEM supplemented with 20% fetal bovine serum (FBS), penicillin and streptomycin culture medium. Finally, cells were counted and plated in poly-D-lysine coated T-75 cm² flasks at the density of 15-18 x 10⁶ /flask. Media was replaced with fresh media 4-5 hours after plating and then every 3 days. After 8 days in culture, flasks were shaken on an orbital shaker for 18-20 hour at 200 rpm to release microglia and oligodendrocytes from the mixed cultures. After shaking, media is completely replaced and washed several times with fresh media. Purified astrocytes were then trypsinized replated on collagen pre-coated plates for assay.

II. Microglia isolation

Microglia were isolated from cortices of postnatal day 2 sprague dawley rat pups using a shaking method. Cortical tissues were first dissected followed by removal of meninges. The
tissues were then briefly centrifuged and washed with cold HBSS. Tissue dissociation was performed by adding papain from Neural Tissue Dissociation Kit (Papain) by Miltenyi Biotec Inc. Tissues were mixed with papain solution using gentle MACs dissociator followed by incubation for 15 min at 37°C. Tissues were further incubated with DNAse for 30 seconds and mixed until well dissociated. After dissociation, cells were passed through 40uM sterile filter and centrifuged at 2000 rpm for 10 min. Later the cells were resuspended into DMEM supplemented with 20% fetal bovine serum (FBS), penicillin and streptomycin culture medium. Finally, cells were counted and plated in poly-D-lysine coated T-75 cm² flasks at the density of 15-18 x 10⁶ /flask. Media was replaced with fresh media 4-5 hours after plating and then every 3 days. After 10 days in culture, flasks were shaken on an orbital shaker for 1 hour at 150 rpm to isolate microglia from the mixed cultures. After shaking, cells are collected, filtered and plated on Poly D lysine pre-coated plates for assay.

III. Neuron isolation

Neurons were isolated from embryonic day 16-18 rodent brains. First we collected the brains in 100cm petridish filled with cold Hank's Balanced Salt Solution (HBSS). Using a dissecting microscope we removed the brainstem and meninges. Then cortices were dissected and collected in 15 ml conical tube with HBSS. Cortical tissues were washed with cold sterile HBSS twice followed by addition of Trypsin (Sigma). Tissues were incubated with trypsin at 37°C for 20 mins. After incubation, DNAse I was added to tissue for 30 seconds. Then tissues were mixed thoroughly and filtered through 70uM cell strainer. DMEM with 10% heat inactivated FBS media was added to the dissociated cells and were counted using
hemocytometer. Cells were plated Poly D lysine pre-coated plates in neurobasal media with B27, glutamax and penicillin streptomycin.

IV. Astrocyte treatment paradigm

Astrocytes were plated onto collagen coated 24 well plates at the density of 100,000 cells/well in DMEM with 20% FBS media for 24 hours. Cells were serum starved before adding compounds. Cells were then stimulated with or without 60 ng/ml LPS (Sigma) in the presence and absence of different doses of GTS21 or PNU282987 to measure cytokine levels.

V. Measurement of secreted TNF using enzyme linked immunosorbent assay (ELISA)

TNF-α secretion upon LPS treatment was measured with α7 nAchR agonist, GTS21 in purified astrocyte cultures. GTS21 pretreatment was done for 1 hour followed by 4-24 hours of LPS treatment.

Following the treatment, cell free culture media was collected and TNF-α and IL6 were measured using a commercially available mouse colorimetric ELISA plates (eBiosciences). Manufacturer’s protocol was followed for performing the assay.

VI. Immunocytochemistry

For immunofluorescence, purified astrocytes were plated onto collagen coated plates. 24 hour post astrocyte plating, cells were fixed with 4% paraformaldehyde for 15 minutes and washed three times with PBS. After fixation, cells were permeablized with 1X GDB buffer (Gelatin Solution, 0.3% Triton X-100, Phosphate Buffer, NaCl). Cells were then incubated with anti-GFAP antibody (Sigma) and anti- NF-κB antibody (Abcam) overnight at 4°C, followed by
washing three times with PBS and 1 hour incubation of Alexa-488 or 594 conjugated secondary antibody (life technology). DAPI (life technology) was used as nuclear stain.

**VII. Immunostaining quantitation**

Images were captured and analyzed using Cellomics ArrayScan XTI high-content analysis system. This system contains high-resolution photometrics X1 CCD, 14 bit camera used for automated image acquisition. The cellomics target activation bioapplication was used for processing and analyzing the images to quantitate the percentage of purity of astrocytes within a well and spot detection bioapplication was used for processing and analyzing the images to quantitate the percentage of NF-κB nuclear translocation within a well.

**VIII. Total RNA extraction from cells**

Total RNA was extracted from astrocytes cultured in 6-well dishes using RNeasy miniprep plus kit from Qiagen according to manufacturer’s instructions. Genomic DNA was eliminated using genomic DNA eliminator column provided with the RNA isolation kit. Purified RNA was quantified using NanoDrop® ND-1000 UV-Vis Spectrophotometer. The quality of RNA was determined by using OD 260/ OD 280 and OD 260/ OD 230 which were approximately 1.8-2. Total RNA was reverse transcribed into cDNA using high capacity cDNA reverse transcription kit (Applied biosystems) using manufacturer’s protocol.

**IX. Total RNA extraction from tissues**

RNA extraction from frozen tissues was performed using QIAzol extraction method. 2.3 mm stainless steel bead was added to each of the frozen tissues collected in the RNase free 96-
well blocks. Tissues were then disrupted by adding QIAzol reagent followed by subjecting the block to Mini-Beadbeater for 4 cycles of 45 seconds each. This results in efficient disruption of the tissues. Aqueous layer was collected after mixing with chloroform. Equal volume of 70% ethanol was added to the aqueous layer, mixed thoroughly and applied to RNeasy 96 plates. Purification of RNA was done according to manufacturer’s protocol.

**X. Real time polymerase chain reaction (rt-PCR)**

Target gene primers along with 6-FAM™ dye-labeled Taqman MGB probe for quantitative PCR analysis were obtained from Applied biosystems. Each reaction contained 100ng of DNA, 900nM each of forward and reverse primers and 250nM Taqman probe. Temperature conditions consisted of a 10min one cycle at 95°C, followed by 40 cycles of 95°C for 0.15min and 60°C for 1min using Stratagene Mx 3005P. All samples were measured in duplicates along with GAPDH as normalizing gene and the no template control was negative for all runs. Final analysis was done using comparative CT method. Amplified samples were then separated on ethidium bromide containing agarose gel. Samples were viewed using Kodak system.

**XI. Gene expression analysis**

TaqMan® OpenArray® Gene Expression platform was used to perform a transcriptomic analysis to comprehensively evaluate inflammatory responses upon α7 nAchR activation in an *in vitro* inflammation model using LPS. Total RNA extracted from astrocytes treated with LPS alone and LPS in the presence of GTS21 were reverse transcribed into cDNA. The cDNA was then loaded to TaqMan® OpenArray® Mouse Inflammation Panel plate (Life Technologies, Ref.
consisting of 632 gene targets selected for their involvement in inflammatory response. QuantStudio 12K Flex Real-Time PCR System (Life Technologies) was used to perform real time PCR and quantitative gene expression. Fold changes (RQ) in expression of inflammatory genes were calculated using comparative CT method. A corrected p-value of 0.05 was used to identify differentially expressed genes.

Next, based on these differentially expressed genes, a gene set enrichment analysis was conducted using the software package GAGE (Generally Applicable Gene-set Enrichment) in R Bioconductor. This software provides a list of Kyoto Encyclopedia of Genes and Genomes (KEGG) mouse pathways that are enriched by the differentially expressed genes. We aimed to identify pathways that are significantly down regulated upon treatment with GTS21. The R package, Pathview, was used to visualize maximally enriched KEGG pathways.

XII. Cell viability assay

LDH (Lactate dehydrogenase) release assay (Promega, CytoTox 96® NonRadioactive Cytotoxicity Assay) was performed on cell free condition media collected after treatment with LPS and α7 nAchR agonists. After treatment, media was collected and centrifuged at 1000 rpm for 5 min. Supernatant was used for the assay. 50 μl of collected media was incubated for 30 minutes at room temperature with reconstituted LDH substrate. Reaction was stopped after addition of stop solution and absorbance was measured at 490 nm on a spectramax plate reader.

XIII. Nitrite Assay

We used the Griess reaction to evaluate the production of nitrite (NO2-), which is a breakdown product of nitric oxide (NO). Astrocytes were treated with LPS and GTS21 for 3
days in DMEM in the absence of phenol red. After the treatment, cell free conditioned media was collected for the assay. This assay is based on a diazotization reaction. First, the media was incubated for 10 min at room temperature with reagent A containing sulfanilamide followed by 10 min incubation with reagent B, N-1-naphthylethlenediamaine dihydrochloride. This results in the formation of colored compound, which was measured at 540 nm using a microplate reader. The amount of color formed is directly proportional to the amount of nitrite levels in the test samples.

**XIV. Neuronal survival assay**

For this assay we added LPS treated astrocyte conditioned media in the presence and absence of α7 nAChR agonists, GTS-21 and PNU282987. Astrocyte conditioned media was treated in cultured neurons for 24 hours. Caspase-3 is a well-recognized marker for apoptosis; therefore, neuronal apoptosis was measured using The CellEvent® Caspase-3/7 green detection reagent. This reagent is a four amino acid peptide (DVED), which is conjugated to a nucleic acid binding dye and is intrinsically non-fluorescent. The DEVD peptide sequence is a cleavage site for caspase-3 and caspase-7, therefore activation of caspases in apoptotic neurons causes cleavage of this DEVD peptide and subsequent binding of dye to the DNA resulting in fluorescence of conjugated dye.

**XV. Protein isolation and western blot analysis**

Cells were scraped in 1x lysis buffer from cell signaling technology with protease and phosphatase inhibitors, DTT and SDS, followed by sonication with F60 Sonic Dismembrator (Fisher Scientific). Amount of protein in each sample was quantified using Pierce BCA Protein
Assay Kit. All samples were diluted to the same concentration. For western blot analysis, samples were boiled at 95 °C for 5 minutes and loaded on Criterion TGX precast protein gels from Bio-Rad. Gels were ran for 1 hour at 150V followed by transferring proteins on to nitrocellulose membrane using an iBlot system from Invitrogen. 5% non-fat dried milk in tris-buffered saline, tween 20 was used to block the membranes for 1 hour. After blocking, primary antibody was incubated overnight at 4°C, followed by washing with 1x- tris-buffered saline, tween 20 for 30 minutes. Secondary antibody was then incubated at room temperature for 45 minutes. After washing the membranes for 30 minutes, Super Signal West Femto Substrate was added to detect horseradish peroxidase (HRP) on the membranes. Chemiluminescence was measured and quantified using Syngene gel imaging system.

XVI. Animals

All procedures involving animals were approved by the Biogen Institutional Animal Care and Use Committee (IACUC), which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. 8-12 weeks old BALB/c Oslo Tg NF-kB RE Oslo BALB/c reporter mice were used for in vivo experiments.

XVII. NF-κB luciferase in vivo and brain ex vivo imaging

Whole body in vivo and brain ex vivo NF-kB luciferase signal was evaluated in images acquired on the IVIS Spectrum instrument (Perkin Elmer, Hopkinton, MA), using the Perkin Elmer proprietary software, Living Image (v4.3.1). Luciferin was injected intraperitoneally at 150 mg/kg. Two minutes after luciferin injection, mice were anesthetized with 2.5% inhaled Isoflurane, and then transferred to the IVIS for in vivo imaging. Throughout imaging, mice were
maintained at 2.5% Isoflurane. Ten minutes after luciferin injection, the optical image was acquired. Image analysis for quantitation of specific regions of interest was performed with Living Image Software. After in vivo imaging, mice were again injected with luciferin for ex vivo brain imaging, which was conducted after euthanizing the animal and harvesting the brain.

**XIX. Data analysis**

Experimental results were analyzed using GraphPad Prism software. Data are expressed as mean (+/- standard deviation). Differences in means for continuous dependent variables were compared using unpaired Student t-tests (for two groups) or one-way ANOVA with adjustment for multiple comparisons (for more than two groups). Two-tailed p values of less than 0.05 were considered as statistically significant.
RESULTS

SPECIFIC AIM 1: To develop, optimize and validate a protocol for culturing astrocytes from rodent brain and evaluate anti-inflammatory properties of astroglial α7 nAchR

I. Preparation of cultured astrocytes

Primary astrocyte cultures were prepared from cortices of C57BL/6 mice at post-natal day 2. Tissue samples were dissociated using papain. Dissociated samples were plated in poly-D-lysine 75 cm² flasks for 7-9 days. Cells were then shaken on orbital shaker for 18-20 hours at 200 rpm to remove microglia and oligodendrocytes. Purified astrocytes were trypsinized and plated for assay. Figure 6 summarizes steps of this protocol.

Figure 6- Culturing protocol for mouse astrocytes

Astrocytes were cultured and isolated from cortices of C57BL/6 mice at post natal day 2
II. Characterization of astrocyte culture purity by immunocytochemistry

To determine the purity of the astrocyte cultures obtained as discussed in section I, we used immunocytochemistry for astrocyte cell specific marker, GFAP. Isolated astrocytes cultures were plated for 24 hours. Next, they were fixed with 4% paraformaldehyde and stained with GFAP. The ArrayScan XTI high-content analysis system with high-resolution photometrics X1 CCD, 14 bit camera was used for automated image acquisition of 20 fields. In each field, two channels were captured to image nuclei, DAPI (blue) and astrocyte marker, GFAP (red).

Figure 7- Astrocyte purity characterization by immunocytochemistry

Purity of astrocyte in our cultures was found to be 90% as measured by the percentage of GFAP positive cells within a well.

The Cellomics Target activation bioapplication was used for processing and analyzing the images to quantitate the percentage of GFAP positive cells within a well. Using this method, the
purity of astrocytes was determined to be 90%. Figure 7 demonstrates cultured astrocytes positive for GFAP.

III. Characterization of astrocyte activation upon treatment with LPS

Lipopolysaccharides (LPS) are known to stimulate inflammatory responses in variety of cells including astrocytes. Therefore we used LPS as inflammatory stimuli to activate astrocytes and characterize their inflammatory response. Primary astrocyte cultures were treated with 60 ng/ml concentration of LPS for 4 hours. Upon activation by LPS, astrocytes release pro-inflammatory cytokines. Using sandwich ELISA, we measured secretion of two pro-inflammatory cytokines: TNF-α and IL6. As shown in Figure 8, we observed statistically significant increase in both these cytokines.

![Graph showing TNF fold change and IL6 fold change](image)

**Figure 8- Effect of LPS treatment on TNF and IL-6 secretion**

LPS (60 ng/ml) treatment for 4 hours activated astrocytes and resulted in significant increase in pro-inflammatory cytokine secretion in astrocytes, * p<0.05 as compared with untreated (t-test)
IV. Characterization of astrocytes by activating them with LPS and measuring nuclear translocation of NF-κB transcriptional subunit p65

To further characterize astrocyte response to LPS, we investigated the subcellular localization of the NF-κB transcriptional subunit p65 upon LPS treatment. Isolated primary astrocytes were activated with 60 ng/ml of LPS for 3 hours and NF-κB localization was detected using immunofluorescence. Image acquisition and analysis was performed using high content image analysis (Cellomics).

Figure 9- NF-κB p65 subunit nuclear translocation with LPS treatment

LPS (60 ng/ml) treatment for 3 hours activated astrocytes and resulted in nuclear translocation of p65 subunit of NF-κB in astrocytes
In each field, three channels were captured to image nuclei, DAPI (blue), astrocyte marker, GFAP (green) and NF-κB (red). As indicated in Figure 9, significant increase in NF-κB nuclear accumulation observed at 3 hours of LPS treatment as compared to untreated cells.

V. Effect of LPS treatment on the morphology of astrocytes

We characterized the standard astrogliosis response using LPS treatment. To examine

![Figure 10- Effect of LPS treatment on the morphology of astrocytes](image)

Stimulation of astrocytes with 60 ng/ml LPS for 24 hours in low serum culture media increased cell processes substantially as compared to the control cells.
astrocytes in the presence of LPS, cells were stimulated with 60 ng/ml LPS for 24 hours in low serum culture media. Post LPS treatment, cells were fixed and stained with astrocyte marker, GFAP shown in green. Fluorescent imaging was performed using the inverted Zeiss microscope. As shown in Figure, LPS modified the morphology of astrocytes by increasing cell processes as compared to the control cells (Figure 10).

VI. α7 nAChR expression in mouse astrocytes using quantitative RT-PCR and fluorescent labelled α-bungarotoxin binding

To evaluate the expression of α7 nAChR in primary astrocyte cultures, we performed quantitative RT-PCR. We first performed total RNA isolation from astrocytes and then convert into cDNA for qRT-PCR analysis using taqman probes. As shown in Figure 11, our results indicate α7 nAChR subunit mRNA expression in cultured astrocytes. There was no genomic DNA detected in this assay shown here by no template and no reverse transcription controls.

<table>
<thead>
<tr>
<th>Astrocytes</th>
<th>No template control</th>
<th>No RT control</th>
</tr>
</thead>
<tbody>
<tr>
<td>α7 nAChR</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 11- α7 nAChR gene expression using RT-PCR

α7 nAChR subunit mRNA expression was noted in cultured astrocytes
Figure 12- Surface binding of fluorescently labeled $\alpha$-bungarotoxin on astrocytes

Pretreatment of mouse astrocytes with 100 uM unlabeled $\alpha$-bungarotoxin ($\alpha_7$ nAChR antagonist) and 200 uM nicotine ($\alpha_7$ nAChR agonist), significantly inhibited binding of fluorescently labeled...
α-bungarotoxin shown by decrease in fluorescence confirming expression of α7 nAchR in these cells.

Next, we evaluated surface binding of a selective α7 nAChR antagonist, α–bungarotoxin, to confirm their expression. Upon treatment of Alexa fluor-488-labeled α-bungarotoxin in astrocytes, we observed surface binding as shown in Figure 12. Pretreatment with unlabeled α-bungarotoxin and nicotine, significantly inhibited binding of fluorescently labeled α-bungarotoxin shown by decrease in fluorescence. These experiments confirm that α7 nAchR is expressed in mouse astrocytes.

VII. Effect of α7 nAchR activation on TNF-α and IL6 secretion in LPS treated cortical astrocytes

We investigated the effect of α7 nAChR activation on the secretion of TNF-α and IL6. In order to do this, we pretreated cortical astrocytes with α7 nAchR agonist, GTS21, for 1 hour followed by 4 hour LPS treatment. To ascertain the dose-response effect of α7 nAchR activation on the secretion of TNF-α and IL6, we used increasing doses of GTS21 (from 3 uM to 100 uM). Levels of IL6 and TNF-α secreted in supernatant were measured using ELISA. Lactate dehydrogenase levels were measured using LDH assay to evaluate cell viability upon LPS and GTS21 treatment. Figure 13 summarizes the experimental protocol.
As hypothesized, LPS caused significant increase in IL6 and TNF-α release in these cells. GTS21 caused dose dependent reduction in both IL6 and TNF-α secretion as compared to LPS treated alone (Figure 14). As shown in the Figure 14, no difference in the cell viability was observed due to treatments.
Figure 14- Effect of \( \alpha_7 \) nAChR activation by GTS21 on cytokine secretion and cell death in LPS activated cortical astrocytes

Pre-treatment with GTS21 (\( \alpha_7 \) nAChR agonist) for 1 hr resulted in a dose dependent reduction in both IL6 and TNF-\( \alpha \) secretion as compared to LPS treated alone and no difference in cell viability was observed in astrocyte cultures. * \( p<0.05 \) compared with LPS (60 ng/ml) treatment (one-way ANOVA with adjustment for multiple comparisons)

We also assessed the effect of another \( \alpha_7 \) nAChR agonist, PNU282987 on protein levels of TNF-\( \alpha \) in LPS treated astrocytes. Pretreatment with PNU282987 showed significant reduction of TNF
secretion as compared to LPS treated alone (Figure 15). This effect was dose dependent. This set of experiments suggests that α7 nAchR activation significantly inhibits pro-inflammatory cytokine secretion in LPS treated cortical astrocytes.

**Figure 15** - Effect of alpha7 nAchR activation by PNU282297 on cytokine secretion

Pre-treatment with PNU282987 (α7 nAchR agonist) for 1 hr resulted in a dose dependent reduction in TNF-α secretion as compared to LPS alone in cortical astrocytes cultures. * p<0.05 compared with LPS (60 ng/ml) treatment (one-way ANOVA with adjustment for multiple comparisons)

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**VIII. Effect of α7 nAchR activation on NF-κB nuclear translocation in LPS activated astrocytes**

It is widely known that LPS treatment causes activation and nuclear translocation of NF-κB. NF-κB translocates into the nucleus and causes transcription of several pro-inflammatory cytokines like TNF and IL6. Therefore, to investigate the mechanism underlying the anti-inflammatory
property of GTS21 in LPS stimulated astrocytes, we decided to study the effect of GTS21 on NF-κB nuclear translocation. Using immunofluorescence we measured nuclear translocation of NF-κB. As shown in Figure 16, treatment of astrocytes with LPS caused robust nuclear translocation of NF-κB. Pre-treatment with GTS-21 caused significant reduction in NF-κB translocation into nucleus. The ArrayScan XTI high-content analysis system was used for automated image acquisition of 20 fields for each tested well at 20x magnification. In each field, three channels were captured to image nuclei, DAPI (blue), astrocyte marker, GFAP (red) and NF-κB (green).

**Figure 16- α7 nAchR activation blocks NF-kB nuclear translocation in LPS treated astrocytes**
Treatment of astrocytes with LPS (60 ng/ml) for 3 hrs caused robust nuclear translocation of NF-κB and pre-treatment with α7 nAchR agonist GTS21 (30 uM) caused significant reduction in NF-κB translocation into nucleus.

The Cellomics Spot detection bioapplication was used for processing and analyzing the images to quantitate the percentage of NF-κB nuclear translocation within a well (Figure 17).
Figure 17- Quantification of blockage of NF-κB nuclear translocation with α7 nAchR activation in LPS treated astrocytes

Pre-treatment with α7 nAchR agonist GTS21 (30 uM) for 1 hr resulted in blocking of NF-κB nuclear translocation by approximately 40%, which was statistically significant at p<0.05 (Student’s t-test). Cellomics array scan was used to capture images. For analysis, identification and validation of primary objects (DAPI channel) was based on user defined validation parameters like object area, shape and intensity. Valid objects entered the selection step and invalid objects were excluded from analysis. In the first step of selection a mask over primary objects was created in channel 2. Further filtering of the objects was done by average and total intensity of target channel. Finally using spots, nuclear NF-κB was measured.

Next, we evaluated the effect of α7 nAchR activation on IkB, which is a negative regulator of NF-κB, in LPS treated cortical astrocytes. Cortical astrocytes were pre-treated with GTS21 or PBS, followed by treatment with LPS for 30 minutes. Following treatment the cells were lysed and the protein level of phosphorylated form of IkB. LPS activated astrocytes shows increased levels of phosphorylated form of IkB, indicating nuclear translocation and activation of NF-κB pathway. However, pre-treatment with GTS21 resulted in reduction of phosphorylated form of IkB (Figure 18).
Figure 18- α7 nAchR activation reduces phosphorylated form of IkB-α in LPS treated astrocytes

Pre-treatment with GTS21 (15 uM and 30 uM) resulted in reduction of phosphorylated form of IkB in LPS (60 ng/ml) treated astrocytes

IX. Determination of the specificity of the anti-inflammatory effects of α7 nAchR in astrocytes using specific antagonist

In order to ascertain whether the anti-inflammatory effects observed by GTS21 are specific to α7 nAchRs, we designed an experiment where we used α7 nAchR specific antagonist, methylycaconitine (MLA). This was done by pretreating the cells for 1 hour with MLA, followed by 1 hour of GTS21 treatment and then cells were activated with LPS. TNF-α and IL6 were measured using ELISA in the cell-free supernatant. Figure 19 shows the experimental design.
Figure 19 - Experimental design for α7 nAchR antagonist and agonist treatment

As demonstrated in Figure 20, we observed that pre-treatment with the α7nAchR antagonist MLA significantly reversed the blockage of IL6 secretion by α7nAchR agonists GTS21 and PNU282987. Further, MLA also robustly reversed the blockage of TNF-α by both these agonists. Therefore, we conclude that the anti-inflammatory effects of GTS21 and PNU282987 are mediated through activation of α7 nAchR.
Figure 20- Specificity of the anti-inflammatory effects of α7 nAchR in astrocytes (pharmacology approach)

Pre-treating the cultured astrocytes for 1-hour with 1 uM α7 nAchR antagonist (MLA) resulted in blockage of anti-inflammatory response, as measured by secretion of pro-inflammatory cytokines TNF and IL6, of GTS21 and PNU282987. * p<0.05 compared with LPS 60 ng/ml treatment, # p<0.05 compared to respective agonist treatment alone (both using one-way ANOVA with adjustment for multiple comparisons)

X. Determination of the specificity of the anti-inflammatory effects of α7 nAchR in astrocytes using receptor knock-down approach

In this experiment, astrocytes were transduced with either lenti short hairpin RNA for α7 nAchR or scrambled shRNA in 6-well plate. Knock-down efficiency was found to be approximately 75% using qRT-PCR with taqman probes specific for α7 nAchR (Figure 21a). Next the cells were treated with LPS (60ng/ml) in the presence and absence of GTS21. Followed by treatment,
RNA was isolated from each condition and inflammatory cytokine levels were measured. Upon knockdown of α7 nAchR, GTS21 did not show reduction in the levels of pro-inflammatory cytokines, TNF and IL1B (Figure 21b and 21c). These findings suggest that anti-inflammatory effects of GTS21 is mediated by its activation of α7 nAchR.
Figure 21- Specificity of the anti-inflammatory effects of α7 nAchR in astrocytes (genetic approach)

Knocking down α7 nAchR expression with lenti short hairpin RNA in astrocytes resulted in blockage of anti-inflammatory response of GTS21 (30 uM), confirming the specificity of this effect

XI. Effect of α7 nAchR activation on TNF-α secretion in LPS treated hippocampal astrocytes

We further investigated the effects of α7 nAchRs activation through GTS21 in hippocampal astrocytes to evaluate whether there are any differences in the responses between hippocampal and cortical astrocytes. For this set of experiments, astrocytes were derived from P2 mouse hippocampi using the protocol described in section I. In hippocampal astrocytes, we measured the levels of IL6 and TNF-α after treatment LPS in the presence and absence of the α7 nAchR agonist, GTS21. We also conducted a dose-response analysis of GTS21. Similar to cortical astrocytes, hippocampal astrocytes also showed significant increase in IL6 and TNF-α secretion upon activation with LPS. GTS21 treatment resulted in a dose-dependent decrease in the secretion of IL6 and TNF-α. These results indicate that activation of α7 nAchRs by GTS21 produces similar response in hippocampal astrocytes (Figure 22).
Figure 22- effects of α7 nAchR activation on TNF secretion in LPS activated hippocampal astrocytes

Pre-treatment with GTS21 (α7 nAchR agonist) resulted in a dose dependent reduction in both in both IL6 and TNF-α secretion as compared to control and no difference in cell viability was observed in hippocampal astrocytes
XII. Effect of α7 nAchR activation on multi-array inflammatory cytokine secretion in LPS treated cortical astrocytes

Cortical astrocytes were pre-treated with 30 uM GTS21 or PBS followed by LPS (60ng/ml) treatment for 24 hours. Next, the cell-free condition media was collected and used for multi-array inflammatory cytokine ELISA for the cytokines; IL1α, IL1β, IL6, TNF-α, IL2, IL4, IL10, IL12, and IL13. Pre-treatment with GTS21 resulted in robust reductions in the levels of pro-inflammatory cytokines IL1α, IL1β, IL6, TNF-α; while no change in the levels of IL2, IL4, IL10, IL12, and IL13 was noted (Figure 23).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Control</th>
<th>LPS (60ng/ml)</th>
<th>GTS21 (30μM)</th>
<th>GTS21 (30μM)/LPS (60ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1α</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL1β</td>
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<td>IL10</td>
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<tr>
<td>IL13</td>
<td></td>
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</tbody>
</table>

Figure 23- Effect of α7 nAchR activation on multiarray inflammatory cytokine secretion in LPS activated cortical astrocytes
Pre-treatment with 30 uM GTS21 resulted in statistically significant reductions in the levels of LPS induced pro-inflammatory cytokines IL1α, IL1β, IL6, TNF-α in cortical astrocytes. * p<0.05 compared to control, # p<0.05 compared with LPS treatment (both using one-way ANOVA with adjustment for multiple comparisons)

XIII. Effect of α7 nAchR activation on RNA levels of inflammatory cytokines in LPS treated cortical astrocytes using TaqMan® OpenArray® Mouse Inflammation Panel

Cortical astrocytes were pre-treated with GTS21 or PBS followed by LPS (60ng/ml) treatment for 24 hours. Next, mRNA was extracted from these cells and reverse transcribed into cDNA for loading to a TaqMan® OpenArray® Mouse Inflammation Panel covering 632 genes specifically targeted for inflammation.

Figure 24 is a volcano plot on a log-log scale with the X-axis showing log of fold change and y-axis showing the negative log of corrected p-values. The area above the grey line is indicative of a statistically significant p-value (<0.05). A majority of the inflammatory genes were upregulated upon LPS treatment compared to control (red dots). Pre-treatment with GTS21 resulted in a substantially lower number of inflammatory genes upregulation as compared to LPS (fewer red dots in the lower panel) indicating a robust anti-inflammatory effect of α7 nAchR activation. Overall, pre-treatment with GTS21 resulted in differential expression in 214 out of the total 632 genes. Some of the important genes that were significantly downregulated with GTS21 treatment are summarized in Table 1.
Pre-treatment of cortical astrocytes with GTS21 followed by LPS (60ng/ml) treatment for 24 hours resulted in a substantially lower number of inflammatory genes upregulation (fewer red dots in the lower panel) indicating a robust anti-inflammatory effect of α7 nAchR activation.
Table 1- Important genes that were significantly downregulated with GTS21 treatment

<table>
<thead>
<tr>
<th>Bdkrb1</th>
<th>Cxcl11</th>
<th>Il21</th>
<th>Tnfsf8</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1s</td>
<td>Cxcl1</td>
<td>Il22</td>
<td>Nos2</td>
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<td>C2</td>
<td>Cxcl12</td>
<td>Il22ra2</td>
<td>Ptx3</td>
</tr>
<tr>
<td>C3</td>
<td>Cxcl5</td>
<td>Il27</td>
<td>Reg3g</td>
</tr>
<tr>
<td>Camp</td>
<td>Cd74</td>
<td>Il9</td>
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<td>Saa1</td>
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<td>Crp</td>
<td>Tnf</td>
<td>Sectm1b</td>
</tr>
<tr>
<td>Ccl20</td>
<td>Csf2</td>
<td>Tnfaip3</td>
<td>Sele</td>
</tr>
<tr>
<td>Ccl20</td>
<td>Cd40</td>
<td>Tnfrsf14</td>
<td>Selp</td>
</tr>
<tr>
<td>Ccl4</td>
<td>Csf3</td>
<td>Tnfrsf9</td>
<td>Mbl2</td>
</tr>
<tr>
<td>Ccl5</td>
<td>Il18bp</td>
<td>Tnfsf10</td>
<td>Mefv</td>
</tr>
<tr>
<td>Ccl7</td>
<td>Il19</td>
<td>Tnfsf11</td>
<td>Orm1</td>
</tr>
<tr>
<td>Ccl8</td>
<td>Il1rn</td>
<td>Tnfsf15</td>
<td></td>
</tr>
</tbody>
</table>

We then performed a gene set enrichment analysis using the software package GAGE (Generally Applicable Gene-set Enrichment) in R Bioconductor based on the 214 differentially expressed inflammatory genes with GTS21 treatment. In this analysis, we identified the KEGG pathways enriched by the downregulation of inflammatory genes in the GTS21 plus LPS treated samples compared to LPS alone treated sample. We observed that the NF-κB was statistically significantly down regulated with GTS21 treatment (p-value 0.04). Other pathways that were down regulated upon GTS21 treatment but did not reach statistical significance were cytokine-cytokine interaction pathway, TNF-signaling pathway, and chemokine signaling pathway. A separate R package, Pathview, was used to visualize the genes that were changed with GTS21 treatment in all four of these KEGG pathways (Figures 25-28).
Figure 25- Genes down regulated with GTS21 treatment in the NF-kB pathway in LPS treated astrocytes
Figure 26- Genes down regulated with GTS21 treatment in the cytokine-cytokine interaction pathway in LPS treated astrocytes.
Figure 27- Genes down regulated with GTS21 treatment in the TNF signaling pathway in LPS treated cortical astrocytes
Figure 28- Genes down regulated with GTS21 treatment in the chemokine signaling pathway in LPS treated astrocytes
XIV. Effect of α7 nAchR activation on the morphology of LPS treated cortical astrocytes

We then looked at morphological changes upon α7 nAchR activation in LPS activated astrocytes as a marker for reactive astrogliosis. Astrocytes were pretreated with GTS21 and stimulated using LPS.

Figure 29- Effect of α7 nAchR activation on the morphology of LPS activated cortical astrocytes
Activation of α7 nAchR with GTS21 (30 uM) or PNU28297 (20 uM) pre-treatment for 1 hr significantly reduced the number of processes in LPS (60 ng/ml) activated astrocytes suggesting reduction in reactive astrogliosis

We observed that in the absence of GTS21 pre-treatment, LPS modified the morphology of astrocytes by increasing cell processes and branches. However, GTS21 or PNU28297 pretreatment significantly reduced the number of processes in activated astrocytes suggesting reduction in reactive astrogliosis (Figure 29).

XV. Effect of α7 nAchR activation on nitrite levels in LPS treated cortical astrocytes

Nitric oxide is another proinflammatory signalling molecule that can result in neurotoxicity. Therefore, nitrite (NO2 –), which is a breakdown product of nitric oxide (NO), measurement using Griess reaction was used as an endpoint. Treatment with GTS21 resulted in dose dependent decrease in the levels of nitrite (Figure 30).

Figure 30- Effect of α7 nAchR activation on nitrite levels in LPS activated cortical astrocytes
Pre-treatment of GTS21 with 7.5, 15, and 30 uM for 1 hr resulted in a dose-dependent decrease in LPS induced levels of nitrites further confirming anti-inflammatory properties of α7 nAchR activation. * p<0.05 compared with control, # p<0.05 compared with LPS, 60 ng/ml treatment (both using one-way ANOVA with adjustment for multiple comparisons)

XVI. Characterization of microglial culture purity with immunocytochemistry and inflammatory response with LPS treatment

Microglia are the resident immune cells of CNS and represents about 10-15% of all cells in the brain. Their major function includes constantly scavenging the CNS for damaged neurons or cellular debris. These cells are responsible for the early control of infections by phagocytosis of foreign material and also release various cytotoxic substances such as nitric oxide and hydrogen peroxide. However, persistent activation of microglia along with sustained secretion of inflammatory mediators is thought to have a harmful effect on neuronal function and survival. Therefore along with astrocytes, I also developed an *in vitro* model of inflammation in microglia.
Figure 31 - Characterization of microglial cultures

Isolated cells were positive for microglia specific marker, IBA1 (green). These cells released pro-inflammatory cytokine TNF upon LPS treatment, which is a known inflammatory stimulus.

After isolation of microglial cultures with a shaking method, we fixed the cells with 4% paraformaldehyde and performed immunocytochemistry for the expression of microglia specific marker ionized calcium-binding adapter molecule 1 (IBA1). Figure 31a demonstrates cultured microglia positive for IBA1 (green). LPS is known to stimulate inflammatory responses in microglia. Therefore we used LPS as inflammatory stimuli to activate microglia and characterize their inflammatory response. Primary microglial cultures were treated with 0.1, 1, and 10 ng/ml concentration of LPS for 4 hours. Upon activation by LPS, microglia release pro-inflammatory cytokines. Using sandwich ELISA, we measured secretion TNF-α. As shown in Figure 31b, we observed statistically significant increase in the secretion of TNF with LPS treatment.

XVII. Characterization of microglia by activating them with LPS and measuring nuclear translocation of NF-κB transcriptional subunit p65

To further characterize microglial response to LPS, we investigated the subcellular localization of the NF-κB transcriptional subunit p65 upon LPS treatment. Isolated primary microglia were activated with 1 ng/ml of LPS for 5 hours and p65 localization was detected using immunofluorescence. Image acquisition and analysis was performed using high content image analysis (Cellomics). In each field, two channels were captured to image nuclei, DAPI (blue), astrocyte marker and NF-κB (red). As indicated in Figure 32, significant increase in p65 nuclear accumulation observed at 5 hours of LPS treatment as compared to untreated.
Figure 32- Nuclear translocation of NF-κB transcriptional subunit p65 in LPS treated microglia

Treating isolated microglial cells resulted in significant increase in nuclear translocation of NF-κB compared with control. * p<0.05 compared with control (Student’s t-test)

XVIII. α7 nAChR expression in microglia using quantitative RT-PCR and fluorescent labelled α-bungarotoxin binding

To evaluate the expression of α7 nAChR in primary microglia culture, we performed quantitative RT-PCR. We first performed total RNA isolation from microglia and then converted into cDNA for qRT-PCR analysis using taqman probes. As shown in Figure 33, our results indicate α7 nAChR subunit mRNA expression in cultured microglia. There was no genomic DNA detected in this assay shown here by no template and no reverse transcription controls.

Next, we evaluated surface binding of a selective α7 nAChR antagonist, α -bungarotoxin. Upon treatment of Alexa fluor-488-labeled α-bungarotoxin in microglia, we observed surface binding as shown in Figure 34. Pretreatment with nicotine, significantly inhibited binding of fluorescently labeled α-bungarotoxin shown by decrease in fluorescence. These experiments confirm that α7 nAchR is expressed in microglia.
Figure 33- α7 nAChR gene expression using RT-PCR

α7 nAChR subunit mRNA expression was noted in cultured microglia (100 ng template)

Figure 34- Surface binding of fluorescently labeled α-bungarotoxin in primary rat microglia
Pretreatment of microglia with 250 uM nicotine (α7 nAchR agonist) significantly inhibited binding of 2 ug/ml of fluorescently labeled α-bungarotoxin shown by decrease in fluorescence confirming expression of α7 nAchR in microglial cultures

**XIX. Effect of α7 nAchR activation on TNF-α and IL6 secretion in LPS treated microglia**

We assessed the effects of α7 nAchR agonists, PNU282987 and GTS21, on protein levels of TNF-α in LPS treated microglia. Pretreatment with these two agonists showed significant reduction of TNF secretion as compared to LPS treated alone (Figure 35). This effect was dose dependent. This set of experiments suggests that α7 nAchR activation significantly inhibits pro-inflammatory cytokine secretion in LPS treated microglia.

![Figure 35- Effect of α7 nAchR activation on TNF and IL-6 secretion in LPS treated microglia](image)

Pre-treatment with α7 nAchR agonists resulted in a dose-dependent reduction in pro-inflammatory cytokine, TNF, secretion indicating a robust anti-inflammatory effect of α7 nAchR
activation in these cells. * p<0.05 compared with LPS 1ng/ml treatment (one-way ANOVA with adjustment for multiple comparisons)

XX. Effect of α7 nAchR activation on NF-κB nuclear translocation in LPS treated microglia

Using immunofluorescence, we measured nuclear translocation of NF-κB in LPS activated microglia. As shown in Figure 36, treatment of microglia with LPS caused robust nuclear translocation of NF-κB. Pre-treatment with α7 nAchR agonist, PNU282987 caused significant reduction in NF-κB translocation into nucleus.

![LPS treatment](image1)

![PNU282987+LPS](image2)

*Red: NF-κB
Arrows indicate cells without nuclear NFκB*

Figure 36- α7 nAchR activation blocks NF-κB nuclear translocation in LPS treated microglia

Treatment of astrocytes with LPS (1ng/ml) caused robust nuclear translocation of NF-κB and pre-treatment with 20 uM α7 nAchR agonist PNU282987 for 1 hr caused significant reduction in NF-κB translocation into nucleus.
The Cellomics Spot detection bioapplication was used for processing and analyzing the images to quantitate the percentage of NF-κB nuclear translocation within a well (Figure 37).

![Nuclear NFkB](image)

**Figure 37- Quantification of blockage of NF-kB nuclear translocation with α7 nAchR activation in LPS activated microglia**

* p<0.05 compared with untreated, # p<0.05 compared with LPS alone (both using one-way ANOVA with adjustment for multiple comparisons)

Microglial cultures have certain limitations including a low yield of cells and available RNA. Therefore, conclusively evaluating effects in these cells is difficult. Further, there are no specific markers to distinguish between microglia and peripheral macrophages, which makes definitely demonstrating microglia specific effects challenging. Therefore we decided to focus on astrocytes for other Specific Aims.
SPECIFIC AIM 2: To study the effect of α7nAchR activation on Nrf2 signaling in astrocytes

I. α7 nAchR activation induced up-regulation of canonical Nrf2 antioxidant genes

For this aim, we used primary cortical astrocyte cultures derived using the same methodology described in Aim 1. We measured expression profiles of number of canonical Nrf2-responsive genes including HO1, TXNRD1, GCLC and OSGIN1 in these cells. To evaluate RNA changes following treatment with α7 nAchR agonists, we used RT-PCR. Comparative Ct method was used to analyze results by normalizing data against housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and control condition. In this study, we collected RNA from astrocytes after 24-hour treatment of LPS with and without pre-treatment with α7 nAchR agonists GTS-21 or PNU282987. Using TaqMan® Gene Expression Assays, we measured the expression of above-mentioned genes.

We observed significant upregulation of HO1, TXNRD1 and GCLC with GTS21 (Figure 38) and PNU282987 treatment (Figure 40) and this effect was blocked in the presence of α7 nAchR antagonist, MLA. We also measured protein levels of HO1, TXNRD1 and NQO1 using western blot and found significant increase with GTS-21 treatment in astrocytes (Figure 39). GTS21 upregulated canonical Nrf2 genes at both RNA and protein level.
Figure 38 α7 nAchR activation induced up-regulation of canonical Nrf2 antioxidant genes-

Pre-treatment with GTS21 (15 uM and 30 uM) for 1 hr resulted in significant upregulation of canonical Nrf2 antioxidant gents HO1, TXNRD1, and GCLC, in astrocyte cultures treated with LPS. α7 nAchR antagonist MLA (1 uM) significantly reduced this effect indicating the specificity of this response. * p<0.05 compared with LPS treatment, # p<0.05 compared with GTS21 30uM (both using one-way ANOVA with adjustment for multiple comparisons)
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LPS (60ng/ml)</th>
<th>GTS 30 μM +LPS (60ng/ml)</th>
<th>MLA+GTS 30μM +LPS (60ng/ml)</th>
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</thead>
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<tr>
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<tr>
<td><strong>NQO1</strong></td>
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<tr>
<td><strong>TXNRD1</strong></td>
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<tr>
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<td></td>
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<tr>
<td><strong>Actin</strong></td>
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Figure 39- α7 nAchR activation induced upregulation of HO1 protein

A significant increase in protein levels of HO1, NQO1 and TXNRD1 upon GTS21 treatment was noted using western blot and this effect was blocked by the 1 uM α7 nAchR antagonist MLA. * p<0.05 compared with LPS 60 ng/ml treatment, # p<0.05 compared with GTS21 30uM+LPS 60ng/ml treatment (both using one-way ANOVA with adjustment for multiple comparisons)
Pre-treatment with 15 uM and 30 uM PNU282987 for 1 hr resulted in significant upregulation of canonical Nrf2 antioxidant genes HO1, TXNRD1, and SRXN1, in astrocyte cultures treated with 60 ng/ml LPS. α7 nAchR antagonist MLA partially reversed this effect indicating the specificity of this response. * p<0.05 compared with LPS 60 ng/ml treatment, # p<0.05 compared with PNU282987 30uM+LPS 60ng/ml treatment (both using one-way ANOVA with adjustment for multiple comparisons)
II. Reversal of α7 nAchR activation induced up-regulation of canonical Nrf2 antioxidant genes and anti-oxidant effects in astrocytes from Nrf2 knockout mice

We used astrocytes cultured from Nrf2 knockout mice for this experiment and observed significant reduction in upregulation of anti-oxidant genes with GTS21 suggesting that these effects are mediated through Nrf2 signaling (Figure 41).

![Graphs showing mRNA expression of HO1, TXNDR1, GCLC, and Osyn1 in WT and Nrf2 K/O astrocytes](image)

**Figure 41** α7 nAchR activation induced up-regulation of canonical Nrf2 antioxidant genes blocked in Nrf2 deficient astrocytes
Pre-treatment with GTS21 resulted in significant upregulation of canonical Nrf2 antioxidant genes HO1, TXNRD1, GCLC, and OSGIN1 in wild type astrocyte cultures treated with LPS, but not in astrocytes from Nrf2 knockout mice confirming the involvement of Nrf2 pathway.

We also repeated this study in the absence of inflammatory stimuli, LPS, to determine the necessity of stressor in activation of α7 nicotinic receptor mediated changes in Nrf2 signaling pathway. We observed that GTS21 treatment by itself up-regulates Nrf2 responsive genes in

**Figure 42- α7 nAchR activation induced up-regulation of canonical Nrf2 antioxidant genes in Nrf2 knockout astrocytes in the absence of LPS**
Treatment with 30 uM GTS21 resulted in significant upregulation of canonical Nrf2 antioxidant
gens HO1, TXNRD1, GCLC, and OSGIN1 in wild type astrocyte cultures not treated with LPS,
but not in Nrf2 knockout mice confirming the involvement of Nrf2 pathway and direct activation
of the Nrf2 pathway even in the absence of a stressor.

Astrocytes from wild type mice and these effects are robustly reduced in astrocytes from Nrf2
knockout mice (Figure 42). This study indicates α7 nAchR agonists directly activate Nrf2
pathway even in the absence of a stressor.

Further we wanted to evaluate the anti-inflammatory effects of GTS21 in the astrocytes from
Nrf2 knockout mice. Cell free conditioned media was collected from astrocytes cultured from
either wild type or Nrf2 knockout mice and treated with LPS in the presence and absence of
GTS21 pre-treatment. Amount of TNF was measured using ELISA.

Figure 43- Anti-inflammatory response of α7 nAchR activation in Nrf2 knockout
astrocytes
In Nrf2 knockout astrocytes treated with LPS, anti-inflammatory effects of GTS21 were almost completely blocked, suggesting an important role of Nrf2 pathway in the anti-inflammatory properties of these receptors.

As observed in the earlier experiments, GTS21 significantly reduced TNF production in LPS treated wild type astrocytes. However, in Nrf2 knockout astrocytes, this effect was robustly blocked. These results indicate that the anti-inflammatory effects of GTS21 are primarily mediated through its activation of Nrf2 pathway (Figure 43).

III. Changes in expression of anti-oxidant genes in astrocytes upon α7 nAchR activation

To investigate the expression of multiple genes related to oxidative stress, we used a panel of gene probes in resting, LPS activated, and GTS21 plus LPS treated astrocytes. This panel, Oxidative Stress RT² Profiler™ PCR Array, allows researchers to evaluate the expression of 84 genes related to oxidative stress. Some of the genes represented in this panel are peroxidases (glutathione peroxidases (GPx) and peroxiredoxins (TPx)), genes involved in reactive oxygen species (ROS) metabolism, such as oxidative stress responsive genes and genes involved in superoxide metabolism such as superoxide dismutases (SOD).

Figure 44a contains a heat map showing changes in the expression of these genes where red is higher expression and green is lower expression. First two panels show the control condition, next two are LPS treated and last two are GTS21 plus LPS treated conditions. From this set of genes, we plotted few that were significantly changed with treatments (Figure 44b). We identified two distinct patterns of response with GTS21 treatment, in the first pattern, genes including Txn1, GCLC, and NQO1, were unchanged with LPS treatment but significantly up regulated by GTS21. In the second pattern, genes including Gdm, Prdx2, and Cat, were
significantly down regulated by LPS, but restored to baseline levels in the presence of GTS21 treatment. Based on these observations, we note that activation of α7 nAchR has a substantial effect on upregulation of many anti-oxidant genes.

Figure 44- Changes in expression of anti-oxidant genes in astrocytes upon α7 nAchR activation
α7 nAchR activation with GTS21 resulted in significant upregulation of various anti-oxidant genes including those downregulated by the inflammatory stimuli LPS and those that are unchanged by LPS.
SPECIFIC AIM 3: To study the effects of astroglial α7nAchR on neuronal survival

I. Effect of astroglial α7 nicotinic receptor activation on neuronal apoptosis

In this experiment, purified astrocytes were treated with LPS in the presence and absence of α7 nAchR agonists, GTS21 and PNU-282987. The cell-free condition media were collected for the treatment in neurons. Conditioned media from activated astrocytes was then added to primary neuronal cultures for 24 hours. Then, we measured the concentration of caspase 3/7, which is a marker for apoptosis, in the astrocyte condition media. In this assay, we observed significant apoptosis of neurons in the presence of LPS treated astrocyte conditioned media. GTS21 (30 uM) and PNU282987 (20 uM) treatment for 1 hr resulted in substantially reduced caspase activation suggesting less apoptosis and this effect was reversed by α7 nAchR antagonist, MLA indicating the specificity of the response (Figure 45).
When astrocyte condition media (ACM) was added to neurons, we noted that α7 nicotinic receptor agonist treated ACM showed significantly lower levels of Caspase 3/7, while this effect was significantly blocked upon addition of 1 uM MLA. * p<0.05 compared with control astrocyte conditioned media

II. Effect of astroglial α7 nicotinic receptor activation on neuronal cell viability

Using the same ACM approach, we assessed cell viability using LIVE/DEAD viability stain in which the live cells were labelled green with calcein AM (excitation wavelength, 488 nM; emission wavelength, 525 nM) and dead cells were labelled red with ethidium homodimer (excitation wavelength, 550 nM; emission wavelength, 575 nM). In this assay, we again noted high neuron death with LPS, which was significantly rescued by treatment with GTS21 (Figure 46). Observations from this assay indicate that astroglial α7 nicotinic receptor activation has a beneficial effect of neuronal survival.
Figure 46- Effect of astroglial α7 nAChR activation on neuronal cell viability

When astrocyte condition media (ACM) was added to neurons, we noted that α7 nicotinic receptor agonist treated ACM showed lower neuron death compared to LPS only indicating a beneficial effect of α7 nicotinic receptor activation on neuronal survival.
SPECIFIC AIM 4: To study the effect of α7nAchR activation in an *in vivo* model of neuroinflammation

I. Selection of optimal dose of LPS for *in vivo* neuroinflammation model

To evaluate the impact of α7 nicotinic receptor activation in an *in vivo* model of neuroinflammation, we used an animal model with LPS inducible NF-κB luciferase reporter mice. A dose-response study was conducted to select the optimal dose of LPS with 5 groups of 5 mice each; one group received intraperitoneal injection of PBS only while four groups received intraperitoneal LPS at 0.8, 1.7, 3.5, and 7 mg/kg doses for 4 hours.

*Figures 47 and 48* show *in vivo* whole body imaging for NF-κB luciferase signal (dorsal and ventral side, respectively). *Figure 49* shows quantification of NF-κB luciferase signaling in specific tissues *in vivo* (lymph nodes, abdomen, spinal cord, and thymus). *Figure 50* specifically shows quantification of NF-κB luciferase signaling in brain *in vivo* and *ex vivo*.

Based on consistently noted inflammatory response observed with *in vivo* and *ex vivo* imaging without any visible toxicity, the LPS dose of 1.7 mg/kg was selected to induce inflammation.
Figure 47 - *In vivo* imaging: NF-κB luciferase signal in whole animal ventral side

In these images, we noted NF-κB luciferase signaling robustly in 1.7, 3.5, and 7 mg/kg doses of LPS.
Figure 48- *In vivo* imaging: NF-κB luciferase signal in whole animal dorsal side

In these images, we noted NF-κB luciferase signaling robustly in 1.7, 3.5, and 7 mg/kg doses of LPS.
Figure 49- Quantification of NF-κB luciferase signaling in specific tissue \textit{in vivo} (lymph nodes, abdomen, spinal cord, thymus)

A dose dependent increase in NF-κB luciferase signaling was seen in each of these tissues upon LPS treatment.
Figure 50- Quantification of NF-κB luciferase signaling in images of brain \textit{in vivo} and \textit{ex vivo}

A dose dependent increase in NF-κB luciferase signaling was seen upon LPS treatment \textit{in vivo} as well as \textit{ex vivo} brains

**II. Effect of α7 nicotinic receptor activation on NF-κB luciferase signal \textit{in vivo}**

To study the effect of α7 nicotinic receptor activation on NF-κB luciferase signal \textit{in vivo}, four groups of 5 mice each were included in this experiment. In the first group, mice were administered with PBS intraperitoneally twice; in the second group, mice were first administered with PBS followed by LPS (1.7 mg/kg) intraperitoneally; in the third group, mice were first administered with GTS21 (5 mg/kg) followed by LPS (1.7 mg/kg) intraperitoneally; and in the fourth group, mice were first administered with GTS21 (25 mg/kg) followed by LPS (1.7 mg/kg) intraperitoneally.
At 4 hours, mice were injected with luciferin and anesthetized followed by whole body imaging both dorsally and ventrally. Figures 51 and 52 shows dorsal and ventral whole body images along with quantification of NF-κB luciferase signaling for all the included animals, respectively. We observed a reduction in the NF-κB luciferase signaling with 5 and 25 mg/kg doses of GTS21 (panel a) and quantification of luminescence suggested a decreasing trend in NF-κB luciferase signaling in GTS21 treated animals compared to LPS only treated animals.

After in vivo imaging, the mice were sacrificed and luciferase signal was also measured ex vivo in brain. In the ex vivo images, we noted a robust reduction in the NF-κB luciferase signaling with GTS21 treatment (Figure 53, panel a) and quantification of luminescence suggested a decreasing trend in NF-κB luciferase signaling in GTS21 treated animals compared to LPS only treated animals (Figure 53, panel b).

After imaging, liver, spleen and brain tissues were collected for RNA analysis of inflammatory cytokines and antioxidant genes. In mice treated with GTS21, significant reductions in gene expression of inflammatory cytokines (TNF-α, IL1B and IL6) were observed in liver and brain. OSGIN1 and HO1, which are genes downstream of Nrf2 signaling and antioxidant in nature, were significantly increased with GTS21 treatment in brain and liver, respectively (Figures 54-Figure 56). Collectively, these results demonstrate a strong anti-inflammatory effect of α7 nicotinic receptor activation in an animal model of neuroinflammation.
Figure 51- *In vivo* imaging: NF-κB luciferase signal in whole animal dorsal side upon LPS and with or without GTS21 treatment

In these images, we noted a reduction in the NF-κB luciferase signaling with 5 and 25 mg/kg doses of GTS21 visually (panel a) and quantifying luminescence suggested a decreasing trend with GTS21 treatment compared to LPS only treated animals (panel b)
Figure 52- *In vivo* imaging: NF-κB luciferase signal in whole animal ventral side upon LPS and with or without GTS21 treatment

In these images, we noted a modest reduction in the NF-κB luciferase signaling GTS21 visually (panel a) and quantifying luminescence suggested a lower signal with 25 mg/kg GTS21 treatment compared to LPS only treated animals (panel b)
Figure 53- Ex vivo imaging: NF-kB luciferase signal in ex vivo brain upon LPS and with or without GTS21 treatment
In these images, we noted a robust reduction in the NF-κB luciferase signaling with GTS21 treatment (panel a) and quantification of luminescence suggested a decreasing trend with GTS21 treatment compared to LPS only treated animals (panel b).

Figure 54- Gene expression of anti-inflammatory cytokines and anti-oxidant genes in liver
After *in vivo* imaging, the mice were sacrificed and liver was collected for RNA analysis. In mice treated with GTS21, statistically significant reductions in gene expression of inflammatory cytokines, TNF-α and IL6, were observed. Expression of HO1, which is a downstream gene of Nrf2 signaling and antioxidant in nature, was significantly increased with GTS21 treatment.

![Graphs showing gene expression](image)

**Figure 55**- Gene expression of anti-inflammatory cytokines and anti-oxidant genes in brain
RNA analysis of brain tissues obtained from mice sacrificed after *in vivo* imaging demonstrated statistically significant reductions in gene expression of inflammatory cytokines, TNF-α, IL1b, and IL6 with 25 mg/kg GTS21 treatment. Expression of OSGIN1, which is a downstream antioxidant gene of Nrf2 pathway, was significantly increased with GTS21 treatment.
Figure 56- Gene expression of anti-inflammatory cytokines and anti-oxidant genes in spleen

RNA analysis of spleen obtained from mice sacrificed after *in vivo* imaging demonstrated statistically significant reductions in gene expression of IL6 with 25 mg/kg GTS21 treatment.
DISCUSSION

In this study, we observed that treatment with α7 nAChR agonists, GTS21 and PNU282987, significantly reduced LPS-mediated secretion of the inflammatory cytokines in a dose dependent manner in astrocytes and this effect was reversed by pharmacological inhibition of α7 nAChR with the antagonist MLA and by knocking down α7 nAChR expression with short hairpin RNA suggesting specificity of the response. Further, we observed that α7 nAChR activation blocked LPS mediated NF-κB nuclear translocation in astrocytes indicating that the observed anti-inflammatory effect may be mediated through NF-κB pathway. We also demonstrated that treatment with α7 nAChR agonists upregulated canonical Nrf2 antioxidant genes and proteins suggesting antioxidant properties of α7 nAChR. Using an astrocyte conditioned media approach; we demonstrated reduction in apoptotic marker caspase 3/7 and neuronal death in cultures treated with GTS21. Finally, in an in vivo neuroinflammation model using LPS in NF-κB luciferase reporter mice, we demonstrated robust reductions with GTS21 treatment in NF-κB activity using whole body imaging and ex vivo brain imaging. We also observed reduction in gene expression of pro-inflammatory cytokines and increase in Nrf2 target genes with GTS21 treatment in liver and brain tissues.

α7 nAChRs have been recognized as a target with major therapeutic relevance in neurodegenerative diseases because of their reported neuroprotective effects and cognitive enhancement properties. A distinct feature of these receptors is that they are widely expressed in neuronal and non-neuronal cells, including astrocytes. Astrocytes are the most abundant cell type in the brain and are increasingly being recognized as important mediators of neuroinflammation and consequent cognitive impairment. However, the specific role of astroglial α7-nAChRs in neuroprotection has only been evaluated in a couple of prior
investigations. In the first study, Liu et al. demonstrated that activation of astroglial α7-nAChRs may provide protection against dopaminergic neurodegeneration by inhibition of MPTP (in vivo)- and MPP+- or LPS (in vitro)-induced astrocyte activation in PD. In a second study, it was demonstrated that activation of α7-nAChRs resulted in reduction in H$_2$O$_2$ induced apoptosis of astrocytes and expression of glial cell-derived neurotrophic factor (GDNF). Both these studies point towards a critical role of astrocytes in neuroprotection conferred by α7 nAChR activation. However, specific signaling mechanisms by which activation of α7-nAChRs in astrocyte provide anti-inflammatory effects have not yet been investigated. Therefore, results from this study elucidating the role of astroglial α7 nAChRs in lowering neuroinflammation and oxidative stress along with the potential signaling mechanisms responsible for these effects add critical data to the body of evidence suggesting the importance of these receptors expressed in astrocytes in promoting neuroprotection.

Using in vitro astrocyte primary cultures and in vivo mouse inflammation model with LPS inducible NF-κB luciferase reporter mice, we demonstrated that blocking the NF-κB inflammation pathway is involved in the anti-inflammatory effects of α7 nAChRs. In primary cultures of astrocytes, we observed that treatment with α7-nAChR agonists resulted in a significant reduction in nuclear translocation of NF-κB and phosphorylated form of I-κB in LPS treated astrocytes. A gene set enrichment analysis further confirmed the involvement of NF-κB pathway in the anti-inflammatory properties of astroglial α7-nAChRs. In the in vivo model, we demonstrated robust reductions in NF-κB activity using whole body imaging and ex vivo brain imaging in animals treated with α7-nAChR agonist, GTS21.

We also noted that treatment with α7 nAChR agonists in astrocytes upregulated canonical Nrf2 antioxidant genes and proteins suggesting antioxidant properties of α7 nAChR.
Interestingly, in astrocytes from Nrf2 knockout mice resulted in robust reduction of not only the anti-oxidant response of α7 nAchR activation, but also the anti-inflammatory response in the \textit{in vitro} inflammation model; suggesting that the Nrf2 and NF-κB pathways may work in concert to mediate the observed anti-inflammatory response of α7 nAchR. Figure 57 summarizes the proposed mechanism of action for the anti-inflammatory and anti-oxidant response of α7 nAchR based on our results.
b)

Cytoplasm

- α7 AchR
- Keap1
- Nrf2

Keap1 → Nrf2

Nucleus

- Nrf2

Up regulation of Antioxidant genes
- HO1
- OSGIN1
- TXNRD1
- NQO1
- GCLC
Figure 57 - The proposed mechanism of action for the anti-inflammatory and anti-oxidant response of α7 nAchR in astrocytes

a) Activation of α7 nAchR results in inhibition of NF-κB pathway shown by reduction in phosphorylation of IκBα, nuclear translocation of NF-κB and robust reduction in proinflammatory cytokines at the level of both protein and RNA. b) Stimulation of α7 nAchR results in activation of Nrf2 pathway. This allows Nrf2 to dissociate from Keap1 and translocate
into the nucleus to induce transcription of genes which are antioxidant and prosurvival in nature. Our data shows significant increase in canonical Nrf2 target genes, HO1, OSGIN1, TXNRD1, NQO1 and GCLC with α7 nAChR agonist treatment in astrocytes. c) Our studies in the astrocytes isolated from Nrf2 knockout mice shows that α7 nAChR agonist not only failed to activate Nrf2 signaling in the absence of Nrf2 but also showed robust reduction in blocking NF-κB pathway suggesting a crosstalk between the two pathways to exert the anti-inflammatory effects of α7 nAChR agonists. It has been previously shown that Keap1 also binds to IKKβ and promotes its degradation. Therefore we hypothesize that after dissociating with Nrf2, Keap1 readily binds to IKKβ thereby promoting its degradation and preventing it to phosphorylate IκBα which is a negative regulator of NF-kB. This results in sequestration of NF-kB in the cytoplasm by binding to IκBα and preventing it to translocate into the nucleus and inhibiting the induction of proinflammatory cytokine expression.

The cross-talk between Nrf2 and NF-κB pathways has been documented in several prior investigations. Keap1, which is a negative regulator protein of Nrf2, is also identified as a binding partner of the p65 subunit of NF-κB. Therefore, upon activation of NF-κB pathway, the NF-κB p65 subunit promotes nuclear translocation of Keap1 from cytoplasm and increased concentration of Keap1 in the nucleus results in functional inactivation of Nrf2. On the other hand, activation of Nrf2 results in higher concentration of its negative regulator Keap1 in the cytoplasm, which in turn binds to IKKβ and prevents the phosphorylation of IkB, p65 NF-κB subunit nuclear translocation and results in diminished NF-κB signaling. Another potential interplay between the two pathways involves CREB binding protein (CBP), which is a transcription co-activator, and capable of binding to both Nrf2 and phosphorylated p65 subunit
of NF-κB.\textsuperscript{102} Since Nrf2 and NF-κB compete for CBP, knocking out Nrf2 results in higher binding of NF-κB /p65 to CBP and consequently higher transcription of inflammatory genes leading a more potent inflammatory response. Our observation of loss of the anti-inflammatory response of α7 nAchR activation in astrocytes from Nrf2 knock out mice is consistent with this hypothesis.

The Nrf2 pathway has received increasing recognition as a major player in the glial α7 nAChRs mediated neuroprotection. Parada et al.\textsuperscript{94} evaluated the potential role of glial α7 nAChRs in protection against ischemic damage induced by deprivation of oxygen and glucose using organotypic hippocampal slice cultures (OHC). They observed that cell death and ROS production both were reduced upon treatment with α7 nAChR agonist, PNU282987. These effects were mediated by activation of HO1 and were reduced substantially upon immunotoxic depletion of microglial cells. These effects were also confirmed in an in vivo model, where α7 nAChR agonist treatment resulted in improved motor skills in a pro-thrombotic stroke model. This effect was substantially diminished in HO1 knock out mice, suggesting an active involvement of Nrf2 pathway in neuro-protection against brain ischemia.

Navarro et al.\textsuperscript{7} took a similar approach and used OHC to study inflammation and oxidative stress induced by treatment with LPS and a mitochondrial inhibitor, antimycin-A. They also observed that treatment with α7 nAChR agonist led enhanced HO1 expression, ultimately resulting in decreased cell toxicity and abnormal protein aggregation. These findings further point toward an active involvement of Nrf2/HO1 axis in protection against oxidative stress in this model. Recently, the same group of researchers evaluated mitochondrial mass and metabolism in α7 nAChR agonist treated primary glial cultures.\textsuperscript{103} They reported that treatment with PNU282987 resulted in increased mitochondrial mass in glial cells. Absence of Nrf2 or
inhibition of HO1 resulted in loss of this effect, suggesting involvement of Nrf2 pathway in increasing glial mitochondrial functionality. Further, treatment with PNU282987 was unable to increase mitochondrial mass when peroxisome proliferator-activated receptor gamma coactivator-1α (PCG-1α), which is a master regulator of mitochondrial biogenesis, was silenced. They postulated that activation of Nrf2 might lead to increased expression of PCG-1α, which in turn results in improvement of mitochondrial mass and functioning. Findings from our study confirm observations made by earlier investigators regarding the importance of Nrf2 pathway in the glial α7 nAChRs mediated neuroprotection. Additionally, our study critically notes that Nrf2 pathway is not only involved in α7 nAChRs mediated anti-oxidant effects, but also in the anti-inflammatory effects. This is the first study to date demonstrating a potential cross-talk between the Nrf2 and NF-κB pathways as a possible mechanism responsible for α7 nAChRs mediated neuroprotection.

Activation of functional α7 nAChRs expressed on astrocytes has been demonstrated to produce rapid currents and increase intracellular calcium levels. After an initial influx of calcium through astroglial α7 nAChR channels, further modulation of calcium signaling occurs through calcium-induced calcium release from intracellular store. Increase in intracellular calcium in astrocytes is reported to regulate synaptic transmission and plasticity. Astrocytes reside in close proximity to neurons and are key components of neural circuits. Recent evidence suggests that calcium signaling cascades in astrocytes result in increased extracellular glutamate levels, thereby shifting the local neural circuit to a slow oscillation state of synchronized neuronal firing critical in memory consolidation and sleep. Taken together, these studies suggest that activation of astroglial α7 nAChRs may play a role in enhancement of cognitive function. Our results showing anti-inflammatory and anti-oxidant properties of astroglial α7 nAChRs suggest
that in pathological states such as neurodegenerative diseases, targeting these receptors can provide additional benefits through neuroprotection.

In conclusion, our results suggest that activating astroglial α7 nAChRs may have a role in neuroprotection by decreasing inflammation and oxidative stress, and therefore could have therapeutic implication for development of disease modifying treatments of neurodegenerative diseases. The anti-inflammatory effects observed with activation of astroglial α7 nAChRs appears to be mediated through an interaction of Nrf2 and NF-κB pathways. Our findings of robust anti-inflammatory and anti-oxidant effects of astroglial α7 nAChRs provide a compelling rationale for future research evaluating this response in specific neurodegenerative disease models to guide development of novel therapeutics targeting these receptors.
FUTURE DIRECTIONS

While our data clearly indicate a significant interaction between the Nrf2 and NF-kB pathways as a potential signaling mechanism for the observed anti-inflammatory effects of α7 nAchRs, there is a need to further investigate specific proteins or enzymes involved in this process. Specifically, the interaction between Keap1 (Kelch-like ECH- associated protein 1) and IKKβ (IκB kinase β ) is of particular interest as this interaction appears to be one of the leading hypothesis explaining our findings. One approach to do this would be to use siRNA depletion of Keap1 to test the hypothesis that unavailability of Keap1 would increase the levels of IKKβ, which in turn would increase nuclear translocation of NF-kB and result in higher inflammation. Therefore, in this paradigm, treatment with α7 nAChR agonists may result in comparatively lower anti-inflammatory response. Another approach could be use of immunoprecipitation technique to pull down IKKβ and Nrf2 with Keap1 antibody to confirm the binding of Keap1 with both these proteins.

Another important area of future research is evaluating whether the anti-inflammatory effects observed with α7 nAchR agonist treatment in vivo are indeed mediated through astroglial α7 nAchRs. For this evaluation, we are in the process of breeding NF-kB luciferase reporter mice to isolate astrocytes from the postnatal day 0-2 pups and investigate if the reduction in the NF-kB luciferase signal in the ex vivo brain of the reporter mice along with robust reductions in the proinflammatory cytokines in the brain tissues treated with α7 nAChR agonist is mediated through their effect in astrocytes. Finally, future research evaluating this anti-inflammatory and antioxidant response of α7 nAChR agonist in specific neurodegenerative disease models including AD and PD where neuroinflammation and oxidative stress are some of the key regulators enhancing neuronal death and exacerbating disease progression. This will be
beneficial in development of novel therapeutics targeting these receptors in combating neurodegenerative diseases.
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