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Role of Microglial α7 Nicotinic Acetylcholine Receptor Positive Allosteric Modulator in Neuroinflammatory Pain Models in Mice

Muzaffar Abbas
South Dakota State University

ROLE OF MICROGLIAL α7 NICOTINIC ACETYLCHOLINE RECEPTOR POSITIVE ALLOSTERIC MODULATOR IN NEUROINFLAMMATORY PAIN MODELS IN MICE

BY

MUZAFFAR ABBAS

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy in Pharmaceutical Sciences degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this dissertation does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Shafiqur Rahman, Ph.D. Date
Dissertation Advisor

Omathanu Perumal, Ph.D. Date
Head, Department of Pharmaceutical Sciences

Dean, Graduate School Date

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CONTENTS

ABBREVIATIONS

ACh	Acetylcholine
ANOVA	Analysis of variance
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BU	Bumetanide
C	Complementary
CD	Cluster of differentiation
CNS	Central nervous system
Ct	Cycle threshold
DAB	Diaminobenzidine
DAPI	4',6'-diamidino-2-phenylindole
DG	Dentate gyrus
DMSO	Dimethyl sulphoxide
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
GABA	Gamma amino butyric acid
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
h	Hour
Iba-1	Ionized calcium-binding adapter molecule 1
i.c.v.	Intracerebroventricular
IDA	Idazoxan
IκB	Inhibitor of κB

IL-1β	interleukin-1 beta
IL-6	interleukin-6
i.p.	Intraperitoneal
KCC2	Potassium-chloride co-transporter 2
KDa	Kilodaltons
LPS	Lipopolysaccharide
M	Molar
min	Minute
MLA	Methyllycaconitine
nAChR	Nicotinic acetylcholine receptor
NE	Norepinephrine
NF-κB	Nuclear factor-κB
NKCC1	Sodium-potassium-chloride co-transporter 1
PAM	Positive allosteric modulators
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
p-CREB	Phosphorylated cyclic adenosine monophosphate response element binding protein
PPAR-γ	Peroxisome proliferator-activated receptor-gamma
s	Seconds
Sal	Saline
S.E.M.	Standard error of mean
TEL	Telmisartan
TLR4	Toll-like receptor 4

TNF	Tumor necrosis factor
TNFR1	Tumor necrosis factor receptor 1
TNFR2	Tumor necrosis factor receptor 2
TQS	3a,4,5,9b-tetrahydro-4-(1-naphthalenyl)-3*H*-cyclopentan[*c*]quinoline-8-sulfonamide
TrkB	Tyrosine receptor kinase B

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ABSTRACT

ROLE OF MICROGLIAL α7 NICOTINIC ACETYLCHOLINE RECEPTOR POSITIVE ALLOSTERIC MODULATOR IN NEUROINFLAMMATORY PAIN MODELS IN MICE

MUZAFFAR ABBAS

2016

Neuroinflammatory pain affects about 1.5% of the United States population. Around 20-40% patients having neurological disorders are affected with neuroinflammatory pain. As a part of the limbic system, hippocampus is known to play a critical role in pain perception and processing, and is densely populated with microglial cells and α7 nicotinic acetylcholine receptors (nAChRs). Given the role of microglial α7 nAChRs in neuroinflammation, the α7 nAChRs have emerged as potential target for neuroinflammatory pain treatment. We hypothesized that microglial α7 nAChRs positive allosteric modulation in the hippocampus will decrease neuroinflammatory pain at behavioral, cellular, biochemical, and molecular level. The primary objective of current study was to examine the role of an α7 nAChR positive allosteric modulator (PAM), TQS, in neuroinflammatory pain and to determine biochemical, cellular, and molecular mechanisms associated with neuroinflammatory pain in the hippocampus using mouse models.

We demonstrated that TQS reduced neuroinflammatory pain-like symptoms, including tactile allodynia and thermal hyperalgesia in mice. Methyllcaconitine, an α7 nAChR antagonist, reversed antiallodynic and antihyperalgesic effects of TQS suggesting the involvement of α7 nAChR. The TQS decreased the expression of ionized calcium-

binding adapter molecule 1 and cluster of differentiation 11b, microglial activation markers, in the hippocampus. We determined that TQS reduced inhibitor of kappa B, a downstream mediator that decreases due to nuclear factor-κB (NF-κB) inactivation, and p-NF-κB p^{65} expression in the hippocampus. In addition, TQS reduced the expression of tumor necrosis factor (TNF) and increased the level of norepinephrine in the hippocampus. We determined that TQS decreased the expression of brain-derived neurotrophic factor (BDNF). Similarly, we determined that TQS reduced the activation of Na-K-Cl cotransporter 1 and increased K-Cl co-transporter 2 expression.

Taken together, our results indicated that TQS decreased microglial TNF and BDNF expression and associated signaling in neuroinflammatory pain involving α7 nAChR and microglial activation. Our findings provided strong and novel evidence that microglial α7 nAChR positive allostetric modulation-mediated signaling regulate behavioral, cellular, biochemical, and molecular mechanisms underlying neuroinflammatory pain. Therefore, targeting microglial α7 nAChR positive allosteric modulation in the hippocampus might be a novel strategy for the treatment of neuroinflammatory pain.

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OVERVIEW

Neuroinflammatory pain is a chronic neurological disorder affecting about 1.5% of the United States population and is associated with microglial activation (Carter and Galer, 2001; Yoon et al., 2012). Various pharmacological ligands that mediate microglial activation cause neuroinflammatory pain development. For example lipopolysaccharide (LPS) causes microglial activation and induces neuroinflammatory pain-like symptoms (Yoon et al., 2012). Fractalkine, the microglial activator, results in development of behavioral signs of neuroinflammatory pain, including tactile allodynia, and thermal hyperalgesia (Milligan et al., 2004). Furthermore, microglial activation modulators, including fluorocitrate (Meller et al., 1994; Watkins et al., 2007), minocycline (Ledeboer et al., 2005; Mika, 2008; Yoon et al., 2012), ibudilast (Ledeboer et al., 2007), and propentofylline (Sweitzer and De Leo, 2011) are effective in neuroinflammatory pain.

As a part of the limbic system, hippocampus has been known for pain processing and perception (Khanna and Sinclair, 1989; Martuscello et al., 2012). Studies have shown that cholinergic nicotinic pathway exists in the hippocampus which modulates microglial activation involving α7 nicotinic acetylcholine receptors (Pavlov and Tracey, 2006; Shytle et al., 2004). Therefore, hippocampal α7 nicotinic acetylcholine receptors have emerged as novel therapeutic targets for neuroinflammatory pain. The α7 nicotinic acetylcholine receptors are metabotropic and are extensively distributed on microglial cells in the hippocampus. The role of α7 nicotinic acetylcholine receptors-mediated signaling in neuroinflammatory pain is less understood at behavioral, cellular, biochemical, and molecular level. Since α7 nicotinic acetylcholine receptors undergo rapid desensitization, therefore, targeting α7 nicotinic acetylcholine receptors through

positive allosteric modulation and associated mechanisms offers an alternative approach to treat neuroinflammatory pain.

Given the significant association of neuroinflammatory pain and microglial activation involving α7 nicotinic acetylcholine receptors positive allosteric modulation, we hypothesized that TQS, an α7 nicotinic acetylcholine receptors positive allosteric modulator, will play a critical role in regulating behavioral, cellular, biochemical, and molecular mechanisms underlying neuroinflammatory pain. Therefore, the primary goal of this dissertation was to examine the functional role of microglial α7 nicotinic acetylcholine receptors positive allosteric modulator in the hippocampus in neuroinflammatory pain using behavioral methods, including tactile allodynia and thermal hyperalgesia and cellular, biochemical and molecular techniques, including immnunofluorescence assay, diaminobenzidine immunohistochemistry, enzyme-linked immunosorbent assay, norepinephrine assay, Western blot analysis, and quantitative real-time polymerase chain reaction in mice.

Chapter 1: General Introduction

1.1. Neuroinflammatory pain

Neuroinflammatory pain is a chronic neurological disorder characterized by hyperalgesia (increased responsiveness to painful stimulus) and allodynia (painful response to normally innocuous stimulus) (Cao and Zhang, 2008; Ji and Wen, 2006; Xu and Yaksh, 2011). The prevalence of neuroinflammatory pain is 1.5% in the United States (Carter and Galer, 2001). Moreover, about 20-40% patients having neurological disorders are affected with neuroinflammatory pain (Borsook, 2012). Previous study showed that neuroinflammatory pain arises due to tissue damage or inflammation (Cao and Zhang, 2008). This exaggerated pain state occurs due to peripheral sensitization and/or central sensitization (Ji and Woolf, 2001).

The neuroinflammatory pain has been shown to be associated with various neurological disorders, including Parkinson's disease, Huntington's disease, Alzheimer's disease, multiple sclerosis, spinal cord injury, diabetic neuropathy, complex regional pain syndrome, post-herpetic neuralgia, and traumatic brain injury (Borsook, 2012). The neuroinflammatory pain shares some common characteristics with neuropathic pain. These include involvement of activated microglia (Scholz and Woolf, 2007; Svensson et al., 2003; Watkins et al., 2001), nuclear factor κB (NF-κB), proinflammatory cytokines, including tumor necrosis factor (TNF) (Junger and Sorkin, 2000; Schafers et al., 2003; Sommer et al., 2001; Taylor and Feldmann, 2009), norepinephrine (NE) (Covey et al., 2000), brain-derived neurotrophic factor (BDNF), potassium-chloride co-transporter 2 (KCC2) (Nomura et al., 2006), and sodium-potassium-chloride co-transporter 1 (NKCC1) (Galan and Cervero, 2005; Pitcher and Cervero, 2010; Price et al., 2009). Thus,

these mediators/transporters may need to be inhibited for the blockade of neuroinflammatory and neuropathic pain conditions.

Various pharmacologicial agents, including anticonvulsants such as gabapentin, and pregabalin, tricyclic antidepressants, including amitriptyline, desipramine, and nortriptyline, selective serotonin norepinephrine reuptake inhibitors such as duloxetine, and venlafaxine, and topical lidocaine have been used as first-line medications for neuroinflammatory pain (Dworkin et al., 2007). Opioids, including tramadol, morphine, fentanyl, and oxycodone have been used as second-line medications that are suitable for first-line use in certain circumstances in neuroinflammatory pain (Dworkin et al., 2007).

Several other medications have shown efficacy for the treatment of neuroinflammatory pain and are also currently used as third line medications. These include antiepileptic medications (e.g., carbamazepine, lamotrigine, oxcarbazepine, topiramate, levetiracetam, zonisamide tiagabine, and valproic acid), antidepressants (e.g., citalopram, and bupropion), mexiletine, and dextromethorphan. Despite the availability of these different treatment options, effective management of neuroinflammatory pain remains limited due to multiple therapeutic challenges. For example, available medications for neuroinflammatory pain have significant limitations, including limited efficacy, and adverse effects. Thus, there is a need to develop new drugs and explore new brain targets, including hippocampus for the treatment of neuroinflammatory pain.

1.1.1. Hippocampus and neuroinflammatory pain

Hippocampus, the part of limbic system and classically known for memory and learning function, has been known for pain perception (Martuscello et al., 2012; Mutso et al., 2012). It has been less systematically studied with regard to neuroinflammatory pain.

Moreover, it is critically involved in altering mood states (Khanna and Sinclair, 1989). This region is known to process both indirect and direct pain inputs. The indirect inputs innervate the hippocampus involving parabrachial and spinothalamic ascending pathways from the periphery (Duric and McCarson, 2006), while hippocampus receives direct inputs from spinal cord (Cliffer et al., 1988; Dutar et al., 1985; Khanna and Sinclair, 1989). Furthermore, hippocampus is known to interact with other brain areas involved in pain processing, including thalamus (Apkarian et al., 2004), prefrontal cortex (Baliki et al., 2006; Baliki et al., 2008; Metz et al., 2009), anterior cingulate cortex (Li et al., 2010), amygdale (Han and Neugebauer, 2005; Ji et al., 2010), and brain stem (Duric and McCarson, 2007; Suzuki et al., 2002) (Figure 1).

Previous studies have indicated hippocampal abnormalities in animal models of pain, including abnormal proinflammatory cytokine production (Al-Amin et al., 2011; del Rey et al., 2011)**,** deficits in long-term potentiation (Kodama et al., 2007), impaired neurogenesis (Terada et al., 2008), and decreased hippocampal volume (Zimmerman et al., 2009). Previous studies have shown that upon activation, microglia release proinflammatory cytokines, including TNF which increases synaptic transmission in the hippocampus (Beattie et al., 2002; Viviani et al., 2003; Woolf and Salter, 2000). This then results in hypersensitivity of neurons in dorsal horn of spinal cord and causes increased pain sensation (DeLeo et al., 2004; Marchand et al., 2005; Sommer and Kress, 2004). The influence of neuroimmune system and associated mechanisms on the expression and release of neuroglial pain mediators within the hippocampus is not known. Therefore, blocking these mediators in the hippocampus might be better therapeutic strategy for neuroinflammatory pain.

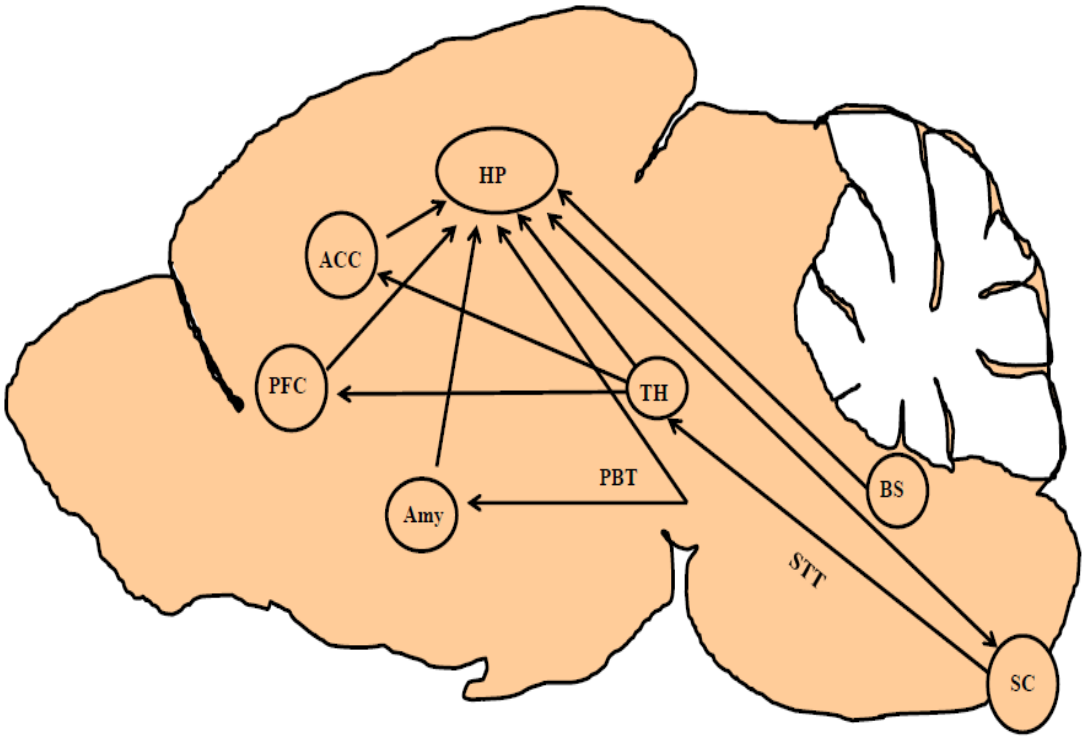

Figure 1: Schematic diagram showing the role of hippocampus (HP) in pain. The HP receives pain inputs from thalamus (TH) through spinothalamic tract (STT). The HP also interact with other areas involved in pan perception/transmission, including anterior cingulated cortex (ACC), prefrontal cortex (PFC), brain stem (BS), and spinal cord (SC). Amygdala (Amy) receives pain inputs from parabrachial tract (PBT) and interacts HP, modulating pain processing and perception. Modified and adopted from Ji et al. (2010) J Neurosci 30:5451-5464; Li et al. (2010) Science 330:1400-1404; Mutso et al. (2012) J Neurosci 32:5747-5756.

1.1.2. Neuroglial cells

Neuroglial cells refer to diverse group of specialized cells that are found both in the peripheral nervous system (perineural glia, satellite glia, and Schwann cells) and central nervous system (CNS) (microglia cells, astrocytes, and oligodendrocytes)

(Kriegstein and Alvarez-Buylla, 2009). These cells constitute about 70% of total cell population in the CNS. Neuroglial cells are non-conducting cells that provide structural support to brain. They help in development, maintenance, repair, supplying biosynthetic products and nutrients to neurons, damaging and removing neurons those undergo death, and finally help in regulating the neuronal microenvironment. Neuroglial cells are further divided into microglial cells which comprise about 5-10% population of glial cells and macroglia which include astrocytes and oligodendrocytes (Moalem and Tracey, 2006).

Among neuroglial cells, microglial cells play a critical role in painful behavior (Alfonso Romero-Sandoval and Sweitzer, 2015). Microglial cells are known as immune system's ambassadors in the CNS and are closely related to macrophages. They fight against infections, and release various mediators that may hypersensitize neurons and play very important role in the mediation of pain (Cao and Zhang, 2008). Astrocytes have been shown to play a critical in pain pathology. The relative contribution of astrocytes versus microglial cells in lipopolysaccharide (LPS)-induced neuroinflammatory pain is not well defined (Guo and Schluesener, 2006; Lee et al., 2010).

1.1.3. Microglial cells and neuroinflammatory pain

Microglial cells, the macrophage-like cells in the CNS, maintain immune surveillance. It is reported that microglial activation happens within minutes (min) (Colton and Wilcock, 2010) but it can be long lasting (Morioka et al., 1991). Microglial cells are either protective or detrimental to neurons depending upon how they become activated. For example, pathology arises when microglial cells are fully activated and their repair efforts fail (Aamodt, 2007). These cells work as a sensors for threats and

considered as first cells that respond to injury (Graeber, 2010; Graeber and Streit, 2010). Microglial cells are known to become activated in neuroinflammatory pain conditions involving NF-κB activation (Alfonso Romero-Sandoval and Sweitzer, 2015; Loram et al., 2010).

Various microglial modulators have shown promising results in preclinical models of pain in the spinal cord. These include fluorocitrate (Meller et al., 1994; Watkins et al., 2007), minocycline (Ledeboer et al., 2005; Mika et al., 2013; Yoon et al., 2012), ibudilast (Ledeboer et al., 2007), and propentofylline (Sweitzer and De Leo, 2011). Fluorocitrate is a microglial metabolic inhibitor. It was the first microglial modulator that was indicated to reduce neuroinflammatory pain (Milligan et al., 2003). Minocycline is tetracycline antibiotic that is shown to inhibit pain behavior by inhibiting microglial activation in different neuroinflammatory pain and neuropathic pain models (Bastos et al., 2007; Cho et al., 2006; Hains and Waxman, 2006; Pabreja et al., 2011). Ibudilast, a phosphodieastrase inhibitor, also inhibits microglial activation and reduces neuroinflammatory pain-like symptoms in different preclinical models (Ellis et al., 2014; Ledeboer et al., 2006). Propentofylline, a methylxanthine derivative and phosphodiestrase inhibitor, also decreases pain-related behavior in animal models by inhibiting microglial activation (Sweitzer et al., 2006; Sweitzer et al., 2001). These findings formed a sound foundation that microglial cells play a critical role in various pain modalities and microglial modulators produce promising results against neuroinflammatory pain.

The term microgliosis (microglial activation) refers to changes in cell morphology, and enhanced expression of microglial mediators, including cluster of

differentiation (CD)11b and ionized calcium-binding adapter molecule 1 (Iba-1) (Alfonso Romero-Sandoval and Sweitzer, 2015; Cao and Zhang, 2008; Ehlers, 2000; Kettenmann et al., 2011; Kreutzberg, 1996; Nakajima and Kohsaka, 2001).

As indicated earlier, microglial activation is characterized by morphological changes. Previous studies have shown that microglial cells adopt resting phenotype in inactivated state but undergo rapid activation in response to acute insults. This results in change in their morphological shape characterized by hypertrophy, retracted processes, and amoeboid morphology under strongly pathological conditions (Chen et al., 2012; Tsuda et al., 2005; Watkins and Maier, 2003; Zielasek and Hartung, 1996).
Bacterial LPS is a well-known toll-like receptor 4 (TLR4) agonist, used to induce microglial activation involving morphological changes (Chen et al., 2012). Although TLR4 antagonists have shown promising results to modulate neuroinflammatory pain-like symptoms (Bettoni et al., 2008), targeting TLR4 for neuroinflammatory pain might be risky due to cancer or infectious disease development (Alfonso Romero-Sandoval and Sweitzer, 2015).

The Iba-1 is 17 Kilodaltons (KDa) protein and is restricted to microglia (Imai et al., 1996). Very little is known about the functions of Iba-1. This protein is known to take part in reorganizing the cytoskeleton within the microglia. Studies have shown that expression of Iba-I is up-regulated when microglial cells become activated (Mori et al., 2000; v Eitzen et al., 1998).Thus increased expression of Iba-I implicates microglial activation. Like Iba-1, CD11b is also microglail activation marker. It is 127 kDa protein and is found be unregulated in neuroinflammatory pain in animal models (Loram et al., 2010). Previous studies showed that CD11b and Iba-1 are not only the mediators up-

regulated during microglial activation but also other mediators, including NF-κB is increased (Loram et al., 2010; Yoon et al., 2012). Taken together, these findings indicate that microglial morphological changes, and up-regulation of Iba-1 and CD11b can be used as markers for microglial activation. Therefore, reducing microglial activation and associated mediators, including NF-κB might be better therapeutic strategy for neuroinflammatory pain treatment.

1.1.4. The NF-κB and neuroinflammatory pain

Until recently, the neuronal dysfunction has been considered to play a critical role in the development of pain hypersensitivity (Watkins et al., 2001; Zhuo et al., 2011). However, recent evidence indicates that microglial cells involving active NF-κB, a transcription factor, are important regulators in the development and maintenance of neuroinflammatory pain (Loram et al., 2010; Watkins et al., 2001; Zhuo et al., 2011). Therefore, NF- κB has emerged as potential target of importance due to its dysregulation in neuroinflammatory pain (Loram et al., 2010).

Previous studies indicate that in resting cells, NF-κB remains in inactivated form by binding to inhibitor of κB (IκB) within the cytoplasm (DiDonato et al., 1996). However, upon appropriate stimulation, such as during inflammation, IκB becomes phosphorylated and is exposed to proteolytic degradation (Gao et al., 2000). The degradation of IκB unmasks NF-κB from inactive to active state. The active NF-κB is then translocated to nucleus and positively regulates the transcription of various pain mediating genes, including IκB (Li and Verma, 2002; Pahl, 1999; Sun et al., 1993) as a part of autoregulatory feedback loop. Therefore, enhanced expression of IκB mRNA parallels increased NF-κB activity and is used as an index for increased NF-κB activation

in neuroinflammatory pain (Ledeboer et al., 2005; Loram et al., 2010; Yoshikawa et al., 2006). Moreover, NF-κB is critically involved in the expression of BDNF and proinflammatory cytokines, including TNF which are critically involved in pain pathophysiology (Ji and Suter, 2007; Loram et al., 2010).

Previous studies have shown that NF-κB inhibitors decrease neuroinflammatory pain-like symptoms. For example, reduction of neuroinflammatory pain was expected as p^{50} (NF-κB) knock-out mice were refractory to induction of acute and chronic arthritis (Campbell et al., 2000). Peripheral administration of NF-κB inhibitor decreases neuropathic and neuroinflammatory pain-like symptoms (Tegeder et al., 2004). Also, intrathecal administration of ammonium pyrrolidinedithiocarbamate and SN50, NF-κB inhibitors, modulate neuroinflammatory pain-like symptoms in animal models (Ledeboer et al., 2005). Taken together, these findings indicate that NF-κB and associated mediators, including BDNF and proinflammatory cytokines such as TNF might be potential therapeutic targets to modulate increased pain responses.

1.1.5. Cytokines

Cytokines are small proteins that constitutively express in precursor form on the cell surface, and can be cleaved to allow release, from where upon they diffuse to act on another cell (Austin and Moalem-Taylor, 2010). Microglial cells are considered as a main source of cytokines in the CNS (Ji and Suter, 2007). Cytokines can be proinflammatory, including TNF, interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-1 beta (IL-1β), interferon-gamma (IFN-γ), interleukin-15 (IL-15), interleukin-17 (IL-17), and interleukin-18 (IL-18); or anti-inflammatory, including transforming growth factor-beta (TGF-β), interleukin-4 (IL-4), and interleukin-10 (IL-10). Among cytokines, TNF is

indicated to be critically involved in neuroinflammatory pain development. Other proinflammatory cytokines involved in producing painful behavior include IL-6, and IL-1β.

1.1.6. The TNF and neuroinflammatory pain

The TNF, a major pro-inflammatory cytokine, has been known to be associated with painful conditions (Austin and Moalem-Taylor, 2010). It is noteworthy to mention that TNF induce its own production involving positive feedback mechanism, acting synergistically to enhance the inflammatory response resulting in chronic inflammation (Watkins et al., 1999). It has been indicated that increased TNF in the hippocampus induces neuroinflammatory pain-like symptoms in animal models (Covey et al., 2000; Ignatowski et al., 1999; Martuscello et al., 2012). Additionally, intracerebroventricular (i.c.v.) injection of recombinant TNF induces neuroinflammatory pain-like symptoms (Covey et al., 2002; Ignatowski et al., 1999; Oka et al., 1996).

The TNF mediates its effects involving constitutively expressed tumor necrosis factor receptor 1 (TNFR1) receptors and inducible tumor necrosis factor receptor 2 (TNFR2) (MacEwan, 2002). TNFR1-mediated signaling is responsible for induction of increased pain sensitivity while TNFR2 activation contributes to maintenance of pain-like symptoms (Austin and Moalem-Taylor, 2010). As mentioned earlier, the binding of TNF to its receptors, results in activation of NF-κB that causes increased production of proinflammatory cytokines and pain hypersensitivity (Ledeboer et al., 2005; Wei et al., 2007).

The TNF-mediated signaling can be modulated either by receptor antagonists, and neutralizing antibodies that result in reversal of neuroinflammatory pain-like symptoms

(Cuellar et al., 2004; Homma et al., 2002; Milligan et al., 2001). For example, etanercept (circulating receptor fusion proteins), and infliximab (neutralising antibody) have been shown to decrease neuroinflammatory pain-like symptoms (Sfikakis, 2010). However, these drugs possess limited lipid solubility and cannot cross blood-brain barrier (BBB). Taken together, these findings indicate that TNF is capable of supporting ongoing inflammation and enhances pain transmission in animal models (Austin and Moalem-Taylor, 2010). Moreover, TNF was shown to influence NE, a monoamine neurotransmitter, release from noradrenergic neurons in the hippocampus (Covey et al., 2000). Therefore, blocking TNF and increasing NE level in the hippocampus might have therapeutic potential for neuroinflammatory pain (Austin and Moalem-Taylor, 2010; Ji and Suter, 2007; Mika, 2008; Romero-Sandoval et al., 2008).

1.1.7. Monoamines neurotransmitters and neuroinflammatory pain

Monoamine neurotransmitters, including serotonin and NE play an important role in pain behavior. Tricyclic antidepressants and selective serotonin norepinephrine reuptake inhibitors used for neuroinflammatory pain have shown to work by increasing serotonin and NE level. Previous studies have shown that NE level is reduced in the hippocampus in pain hypersensitivity (Ignatowski et al., 1999). Moreover, NE and TNF are signals of foremost importance that play very important role in painful behavior, affecting each other level in the hippocampus. For example, the expression of NE decreases as TNF increases in the hippocampus. Moreover, NE increases as TNF is lowered in the hippocampus. Furthermore, increasing NE may alleviate pain sensitivity in animal models. For example, it was shown that tricyclic antidepressants decrease pain by reducing TNF expression and increasing NE level in the hippocampus (Reynolds et al.,

2004). Taken together, these findings indicate that NE is critically involved in pain hypersensitivity. The effects of microglial activation and associated pain mediators, including TNF on NE level in the hippocampus are not known. Therefore, increasing NE level in the hippocampus might have therapeutic utility for painful behavior involving neuroimmune mechanisms.

1.1.8. The BDNF and neuroinflammatory pain

The BDNF plays a critical in neuroinflammatory pain development (Coull et al., 2005; Groth and Aanonsen, 2002). It is produced by microglial cells in neuroinflammatory pain involving NF-κB activation (Ji and Suter, 2007). Moreover, BDNF is proposed to increase pain behavior due to increased expression of NKCC1 and decreased KCC2 expression (Price et al., 2005). A pervious study has shown that expression of BDNF is significantly increased in animal model of spinal nerve ligation (Li et al., 2006) mediated by activated microglial cells (Coull et al., 2005; Cragg et al., 2010). Microinjection of BDNF in CNS results in increased pain behavior in animal models (Zhou et al., 2000). Furthermore, intraplantar injection of BDNF also results in increased pain like symptoms in animal models (Shu and Mendell, 1999). Anti-BDNF antibodies have been known to attenuate pain behavior in animal models (Quintao et al., 2008). Moreover, administration of tyrosine receptor kinase B (TrkB) antibody also reduced development of pain hypersensitivity (Yajima et al., 2002). Additionally, sequestering BDNF using TrkB-Fc fusion protein also reduced development of pain symptoms. Furthermore, knockdown of BDNF using siRNA prevents development of pain behavior (Coull et al., 2005). Therefore, eliminating BDNF suppresses neuroinflammatory pain in animal models (Zhao et al., 2006). Together, these findings

indicate the possibility that BDNF might be required for neuroinflammatory pain development in animal models and blocking microglial BDNF and targeting associated proteins, including NKCC1, and KCC2 could be better strategy for the treatment of neuroinflammatory pain.

1.1.9. The KCC2 and neuroinflammatory pain

The KKC2, a neuronal protein transporter, is responsible to maintain intracellular Cl^- concentration within neuron involving outward movement (Payne et al., 2003). Other transporter involved in maintaining Cl^- concentration within neuron is NKCC1. It was found that reduction in the expression of KCC2 results painful behavior (Coull et al., 2003). Previous study showed that down regulation of KCC2 plays a critical in neuroinflammatory pain development (Cramer et al., 2008; Zhang et al., 2008). Knockdown or blockade of KKC2 transporter induces neuroinflammatory pain-like symptoms confirming that KKC2 is critically involved in neuroinflammatory pain development (Price et al., 2005). Collectively, these findings indicate that reduced KCC2 expression is likely to be an important feature of painful condition and increasing KCC2 expression might be novel therapeutic strategy for neuroinflammatory pain.

1.1.10. The NKCC1 and neuroinflammatory pain

Electrical signaling through neurons is dependent on transporters, including NKCC1 which like KCC2 is also responsible to maintain ion gradient (Payne et al., 2003). NKCC1 is of particular interest with respect to neuronal chloride homeostasis and is predominately expressed on neurons (Plotkin et al., 1997). The NKCC1 is involved for the movement of Cl^- into the cell (Haas, 1994; Plotkin et al., 1997). Research indicates that NKCC1 plays a critical role in the pathophysiology of neuroinflammatory pain. For

example, the expression and activity of NKCC1 increases in neuoinflammation resulting in increased pain sensitivity (Galan and Cervero, 2005; Payne et al., 2003; Price et al., 2005; Valencia-de Ita et al., 2006). This probably happens due to increased influx of Cl^- within the neurons resulting in gamma amino butyric acid (GABA)-mediated dis-inhibition and increase pain sensitivity. Knockout mice have been shown to exhibit reduced neuroinflammatory pain-like symptoms (Sung et al., 2000). Furthermore, phosphorylation of NKCC1 (active NKCC1) results in increased pain behavior (Galan and Cervero, 2005) . Thus activated NKCC1 has been found to induce neuroinflammatory pain-like symptoms in animal models (Lee et al., 2014). Taken together, these studies show that NKCC1 is critically involved in pain hypersensitivity and blocking NKCC1 using nicotinic acetylcholine receptors (nAChRs) based neuro-immune modulators might be a novel target for neuroinflammatory pain treatment.

1.2. Brain nAChRs

Brain nicotinic nAChRs are ionotropic ligand gated cationic channels (Corringer et al., 2000; Le Novere et al., 2002). The opening of nAChR channel is controlled by acetylcholine (ACh) or nicotinic ligands. The nAChRs are permeable to both K^+ and Na^+ ions. Moreover, they are also permeable to Ca^{+2} ions which is affected by the composition of subunits (Gotti et al., 2007). In CNS, nAChR are mainly presynaptic and play a critical role in the release of neurotransmitters, including dopamine, NE, GABA, and glutamate (Gotti et al., 2007). However, they are also present post-synaptically where they mediate fast synaptic transmission (Dajas-Bailador and Wonnacott, 2004; Gotti and Clementi, 2004; Jensen et al., 2005). Furthermore, they are also present on non-neuronal cells, including microglial cells where they modulate neuroinflamatory response (Fucile,

2004; Pocock and Kettenmann, 2007; Shytle et al., 2004). The interaction of nicotinic ligands with nAChRs, results in arousal, fatigue, locomotion, sleep, anxiety, cognition, and central processing of pain (Abbas and Rahman, 2016; Champtiaux and Changeux, 2002; Drago et al., 2003; Gotti and Clementi, 2004; Hogg and Bertrand, 2004; Hogg et al., 2003; Picciotto et al., 2001; Picciotto et al., 2000).

The nAChRs are pentameric heterogeneous comprised of three β (β2-β4) and nine α (α2-α10) subunits encoded by different genes. They consist of five homologous subunits with four binding sites (heteropentameric; α7α8, α9α10) or two binding sites in receptor molecule located between an α and a β subunit (α(α2– α6)-β(β2– β4)). Other pentameric nAChRs are comprised of identical subunits with five similar ACh binding sites in a receptor molecule (homopentameric; α7, α8, and α9) with a central pore (Corringer et al., 2000; Le Novere et al., 2002).

The nAChRs are classified into two major classes, including α-bungarotoxin-sensitive, and α-bungarotoxin-insensitive receptors. The α-bungarotoxin-sensitive receptors which bind with α-bungarotoxin with high affinity consist of α7, α8, α9 and/or α10 subunits. However, the α-bungarotoxin-insensitive receptors bind with nicotine and other nicotinic agonists with high affinity, comprised of α2–6 and β2–4 subunits (Gotti et al., 2006). In case of α-bungarotoxin-sensitive receptors, ACh binds either between two identical subunits (α7, α8, α9) or different α subunits (α9–α10 or α7–α8). However, in case of α-bungarotoxin-insensitive receptors, ACh binds between a α subunit and a β subunit (Gotti et al., 2006). The use of asterisk (*) indicates the presence of nAChR subunit that is unidentified.

The nAChRs are widely distributed in the brain with α4β2 comprising about 90%. The α4β2 receptors are distributed in cortex, hippocampus, striatum, amygdala, ventral tegmental area, substantia nigra, thalamus, cerebellum, and hypothalalus. The α3β4 are widely distributed in adrenal medulla, autonomic ganglia, dorsal medulla, medial habenula, retina, and pineal gland (Gotti and Clementi, 2004). The α7 nAChRs are highly expressed in the hippocampus, cortex, and sub cortical regions of brain limbic system and at low levels in the basal ganglia and thalamic regions (Gotti et al., 2007; Gotti et al., 2006). The α9 and α10 subunit containing receptors have been found in dorsal root of gaglion, and pituitary gland in neurons and non-neuronal sites. Previous studies have shown that α7 nAChRs are expressed on microglial cells and play a critical role in pain control associated with neuro-inflammation (Abbas and Rahman, 2016; Pocock and Kettenmann, 2007; Vincler et al., 2006). Therefore, targeting microglial α7 nAChRs and associated signaling cascades might be better therapeutic strategy for pain control.

1.2.1. The α7 nAChRs

The α7 nAChRs are homomeric with five identical ACh binding sites (Drisdel and Green, 2000) (Figure 2). They belong to neurotransmitter-gated ion channel super family of receptors (Lukas et al., 1999). They are pentameric in nature (Drisdel and Green, 2000), highly permeable to calcium and are undergo desensitization within milliseconds on agonist binding (Couturier et al., 1990; Shen and Yakel, 2009). The α7 nAChRs are not only expressed in neurons but also on non-neuronal cells, including microglial cells in CNS (Pocock and Kettenmann, 2007) and involve different signaling pathways (Grybko et al., 2010; Wang et al., 2003).

A previous study showed that anti-inflammatory reflex exists in the brain (de Lucas-Cerrillo et al., 2011; Pavlov and Tracey, 2006; Shytle et al., 2004) mediated through microglial α7 nAChRs to reduce neuroinflammation (Kawashima et al., 2012; Mencel et al., 2013; Shytle et al., 2004). The activation of microglial α7 nAChRs results in reduced production of proinflmammatory cytokines (Christie and Turnley, 2012; Pena et al., 2010).

It is noteworthy to mention that cholinergic transmission in the brain is impaired by various neurodegenerative diseases. The weakness of cholinergic system facilitate the development of neuroinflammation specially by decreasing the ACh input to microglial cells (Carnevale et al., 2007). Therefore, the α7 nAChR agonists are now considered as a target for neuroinflammation and pain in animal models (Damaj et al., 2000; de Jonge and Ulloa, 2007; Rowley et al., 2010; Wang et al., 2005). However, due to rapid desensitization of α7 nAChRs because of agonist binding at othosteric site, microglial α7 nAChRs positive allosteric modulation could be potential approach for novel analgesic development.

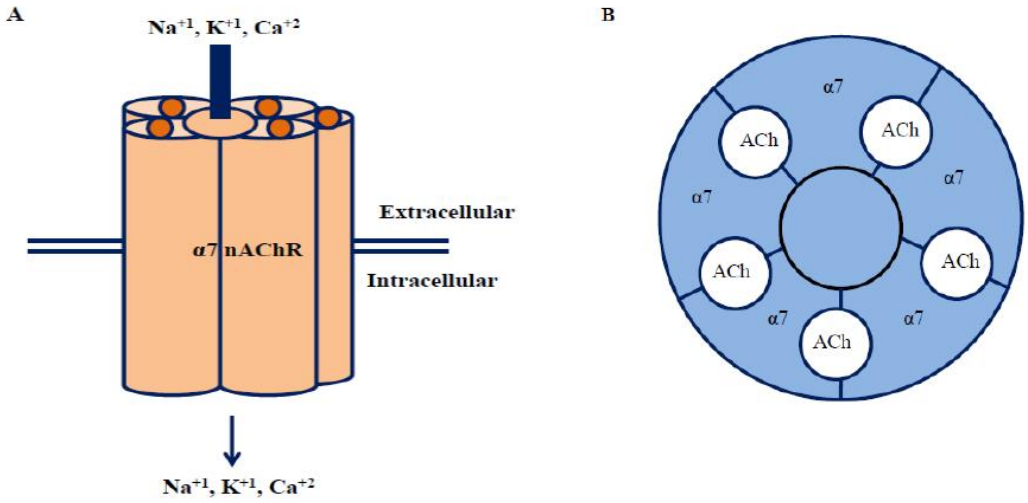

Figure 2: Structure and organization of α7 nicotinic acetylcholine receptors (α7 nAChR). (A) Pentameric arrangement of five identical α7 nAChR subunits arranged around central pore. (B) Acetylcholine binds at each subunit and causes the increase in the intracellular level of Na^{+1}, K^{+1}, Ca^{+2}. Modified and adopted from Gotti and Clementi (2004) Prog Neurobiology 74:363-396.

Unlike, neuronal α7 nAChR, microglial α7 nAChRs are metabotropic receptors (Suzuki et al., 2006). The microglial α7 nAChRs differ from neuronal α7 nAChR in terms of downstream signaling (Shytle et al., 2004; Suzuki et al., 2006). The activation of microglial α7 nAChR causes the activation of phospholipase C and increased release of Ca^{+2} from intracellular stores sensitive to inositol trisphosphate (Suzuki et al., 2006). This then reduces NF-κB activation (Conejero-Goldberg et al., 2008; Egea et al., 2015; Loram et al., 2010; Wang et al., 2004) involving inhibition of IκB degradation (Loram et al., 2010; Tracey, 2009). The reduced activity of NF-κB decreases transcription of proinflammatory cytokines and BDNF (Figure 3). For example, nicotine has been indicated to inhibit NF-κB translocation into the nucleus in LPS-stimulated RAW 264.7 macrophages (Wang et al., 2004). Taken together, these findings indicate that microglial α7 nAChR activation reduces proinflammatory cytokine and BDNF expression.

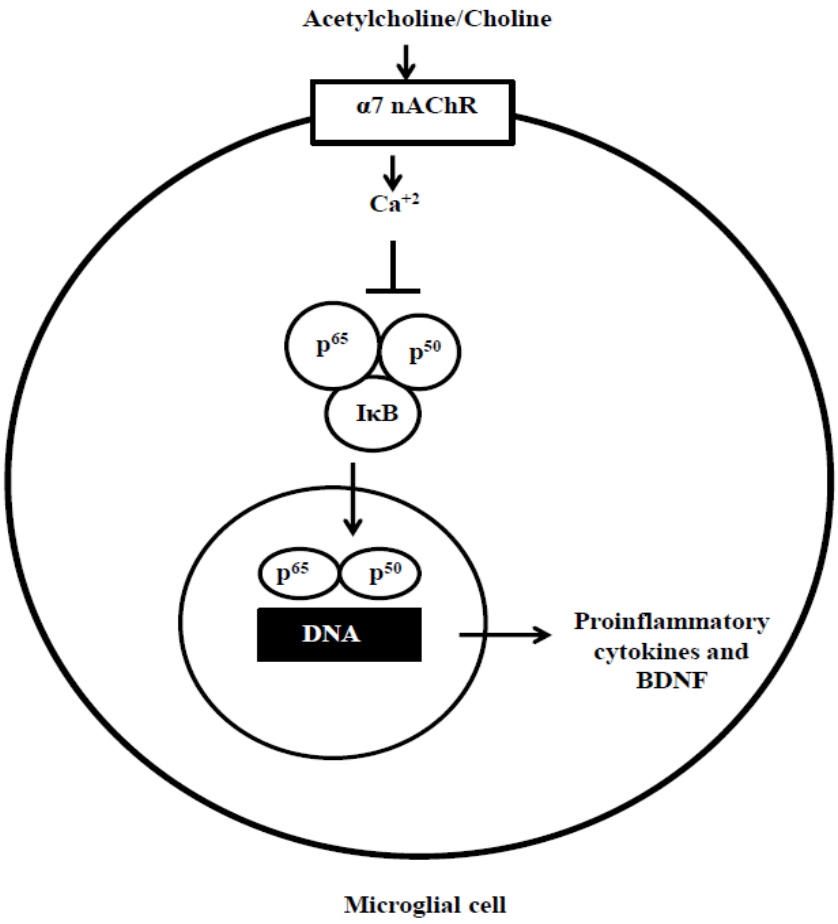

Figure 3: Schematic illustrating the signaling cascade activated by α7 nicotinic acetylcholine receptors (nAChRs) in microglia. Acetylcholine/choline activates α7 nAChRs resulting in increased intracellular Ca^{+2} which in turn reduces the degradation of IκB thus inhibiting the translocation of p^{65} into nucleus. This results in the inhibition of proinflammatory cytokine and brain-derived neurotrophic factor (BDNF) production. Modified and adopted from Egea et al. (2015) Biochem Pharmacol 97:463-72.

The nAChRs have been known to be involved in the process of mediating pain. The analgesic effects of nicotine were reported as early as 1932 (Bannon et al., 1998). The microinjection of nicotine into different regions of the brainstem produced analgesic effects (Umana et al., 2013). The analgesia produced by nicotine was further shown to be

inhibited by the administration of the general nAChR antagonist, mecamylamine (Sahley and Berntson, 1979). However, nicotine was not developed as anlagesic due to less analgesic potential, low intrinsic activity, and side effects (Qian et al., 1993).

Apart from nicotine, the Ecuadorian frog skin-derived alkaloid epibatidine, which acts as a nonselective agonist for nAChRs, was shown to have analgesic properties. Epibatidine was able to reduce pain in the mouse straub tail model (Garraffo et al., 2009). Upon further investigation, it was shown that epibatidine caused antinociception in animal models of pain, it also produced several major adverse effects, including dose-dependent decreases in locomotor activity, and body temperature. Moreover, since epibatidine is a nonselective agonist, it activates multiple subtypes of the neuronal nAChRs, as well as the neuromuscular receptor, which accounts for other side effects, including neuromuscular paralysis, hypertension, and seizures at a dose closer to that produces antinociceptive effects (Sullivan et al., 1994) limiting its use as a therapeutic agent. Based upon these observations, the research focus has since shifted to develop compounds that would selectively target specific nAChR subtypes such as microglial α7 nAChR with the purpose of maintaining the analgesic effect while reducing or eliminating the adverse effects that are seen with nonselective agonists such as epibatidine.

Compounds that involve α7 nAChRs agonism have also been studied for their effects on pain behavior because of their distribution in pain pathways (Seguela et al., 1993). Choline, a by-product of ACh enzymatic degradation by acetyl cholinesterase and a selective agonist for the α7 receptors, has analgesic effects in rodent models of pain (Loram et al., 2010; Wang et al., 2005). Systemic administration of an α7 nAChR

agonist, TC-7020, reverses neuropathic pain in animal models (Loram et al., 2012). A previous study showed that i.c.v. administration of citicoline, precursor for choline, was effective in inflammatory pain models (Bagdas et al., 2011). However, α7 nAChRs undergo rapid desensitization on agonist binding in neuroinflammatory pain where continuous stimulation of microglial α7 nAChRs is needed to inhibit neuroinflammation. The mechanism through which α7 nAChRs undergo desensitization is not known. One alternative approach to prevent desensitization of α7 nAChR is by the use of α7 nAChR positive allosteric modulators (PAMs).

1.2.2. The α7 nAChR PAMs

The term allosteric modulation indicates that protein can exist in various multiple conformational states and binding of allosteric compound changes the energy barrier between these states. For example, ACh binds at interface between subunit protomers (othosteric site) while a molecule that binds elsewhere on the protein works as allosteric modulator thus stabilizing the protein in preferential state (Changeux and Edelstein, 2005). For example, in case of α7 nAChR, the allosteric compound stabilizes the protein in active open state. PAMs increase the gain of the physiological response without having an inappropriate tonic response. In other words, PAMs enhance the activity induced by agonists and exhibit no intrinsic activity (Abdrakhmanova et al., 2010; Arias, 2010; Bertrand and Gopalakrishnan, 2007).

The α7 nAChR function can be modulated by endogenous peptide, including SLURP-1 (secreted mammalian Ly-6/uroquinase plasminogen activated receptor related protein 1) which works as endogenous PAM of α7 nAChRs (Atzori et al., 2016). The PAMs have been classified as either type I, such as NS1738 (Arias, 2010; Haydar and

Dunlop, 2010; Mazurov et al., 2011; Williams et al., 2011), or type II, such as 3a,4,5,9b-tetrahydro-4-(1-naphthalenyl)-3*H*-cyclopentan[*c*]quinoline-8-sulfonamide (TQS) (Arias, 2010; Gill et al., 2011; Gronlien et al., 2007) on the basis of their effects on desensitization (Bertrand and Gopalakrishnan, 2007; Timmermann et al., 2007). The PAMs with little or no effect on desensitization are classified as type I PAMs and those that cause a dramatic reduction in agonist-induced desensitization of α7 nAChR are known as type II PAMs (Chatzidaki et al., 2015). Therefore, type II PAMs modifies the equilibrium between active and desensitized state that result in significantly prolonged responses (Gronlien et al., 2007; Hurst et al., 2013; Hurst et al., 2005; Roncarati et al., 2008b). Furthermore, type II PAMs promote the activation of receptors that are previously desensitized (Hurst et al., 2005).

The TQS is a selective α7 nAChR type II PAM (Arias, 2010; Gronlien et al., 2007). Being lipophilic compound, TQS is expected to cross BBB. The TQS alone has no intrinsic activity at α7 nAChR and is a potent α7 nAChR PAM when co-applied with ACh (Gill et al., 2012; Gill et al., 2011; Gronlien et al., 2007). Furthermore, TQS is known to facilitate the recovery of complete agonist-induced desensitization of α7 nAChRs (Couturier et al., 1990; Gill et al., 2012; Gill et al., 2011) (Figure 4). The effectiveness of TQS in neuroinflammatory pain has not been investigated yet.

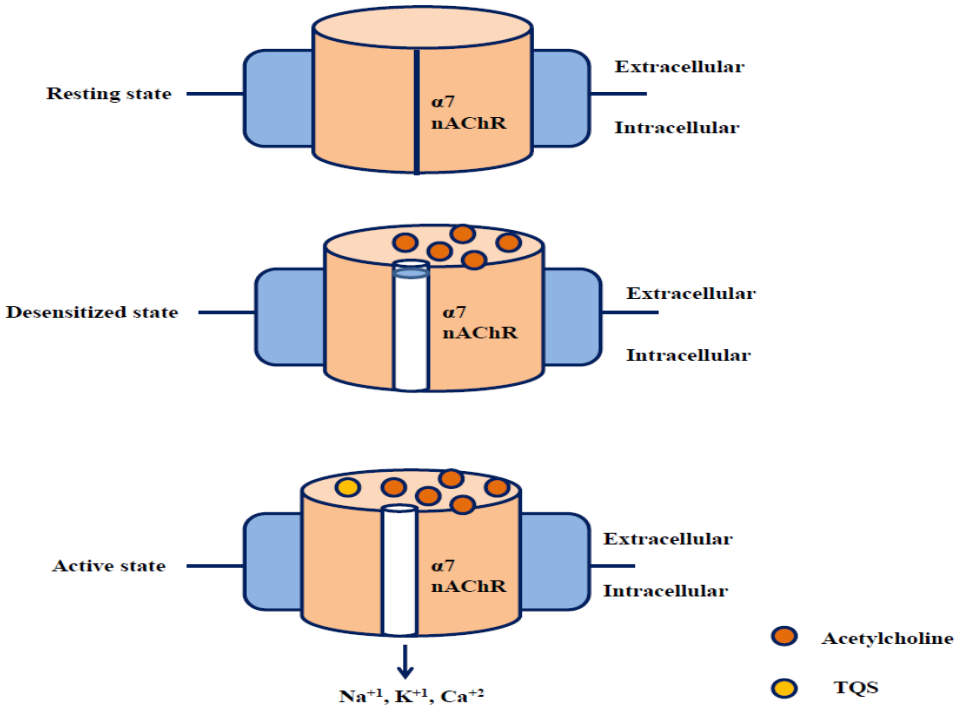

Figure 4: Schematic representation α7 nicotinic acetylcholine receptor (nAChR) desensitization and role of TQS. Binding of α7 nAChR ligand i.e acetylcholine/choline causes the desensitization of α7 nAChR. TQS binds at allosteric site of α7 nAChR and prevents the desensitization induced by acetylcholine/choline. Modified and adopted from Hurst et al. (2013) Pharmacol Ther 137:22-54.

Due to receptor desensitization by α7 agonists, research has focused on α7 nAChR PAMs (Papke et al., 2009). Studies have reported that α7 nACh PAMs facilitate endogenous neurotransmission, and enhance the potency and efficacy of an agonist without directly stimulating the orthosteric site. Thus, these compounds augment and synergize orthosteric site-mediated signaling of endogenous neurotransmitters, including choline, and ACh. In principle, PAMs do not exhibit intrinsic activity at the receptor; however they can increase endogenous cholinergic neurotransmission. These PAMs are

shown to increase maximal efficacy and the potency of endogenous (choline or ACh) or exogenous agonists for the α7 nAChRs. A variety of α7 nAChR PAMs have been evaluated in preclinical models for pain. For instance, PNU-120596, an α7 nAChR PAM, resulted in a significant reduction in mechanical hyperalgesia in rats (Munro et al., 2012). Additionally, PNU-120596 is able to decrease formalin-induced pain by alone, and in combination with the α7 nAChR agonists, including choline, nicotine, and PHA-543613 (Freitas et al., 2013). Another α7 nAChR PAM, NS-1738, has also been tested for its effects on pain. Both NS-1738 and PNU-120596 were able to reduce heat-induced hyperalgesia (Freitas et al., 2013). However, the effects of α7 nAChR PAMs on microglial activation and associated signaling in the hippocampus are not known. Therefore, we hypothesized that α7 nAChR positive allosteric modulation in the hippocampus will be novel therapeutic strategy for neuroinflammatory pain treatment.

1.3. Rationale and objectives

Microglial cells play a critical role in neuroinflammatory pain development and maintenance in the CNS. As described earlier, the microglial cells become active involving NF-κB activation in neuroinflammatory pain. The NF-κB-mediated gene expression causes the release of pain mediators, including TNF, and BDNF. The TNF in turn affects the release of NE while BDNF is involved in the regulation of NKCC1 and KCC2 in pain pathology. Previous studies have shown that cholinergic nicotinic pathway exists in the hippocampus which modulates microglial activation involving α7 nAChRs (Pavlov and Tracey, 2006; Shytle et al., 2004). As a part of the limbic system, the hippocampus is known to play a critical role in pain perception and processing. The effects of microglial α7 nAChR on microglial activation and associated pain mediators in

the hippocampus are not known in neuroinflammatory pain. Since α7 nAChRs undergo rapid desensitization, we hypothesized that targeting α7 nAChRs through positive allosteric modulation (i.e. at allosteric binding site) and associated mechanisms will be a better strategy to treat neuroinflammatory pain.

The overall goal of this dissertation is to determine the role of α7 nAChR PAM in neuroinflammatory pain. The primary objectives of research presented in this dissertation include

i. To determine the effects of α7 nAChR ligand on neuroinflammatory pain and microglial activation in neuroinflammatory pain.
ii. To elucidate the effects of α7 nAChR ligand on NF-κB activation in the hippocampus in neuroinflammatory pain.
iii. To examine the effects of α7 nAChR ligand on TNF expression and NE level in the hippocampus in neuroinflammatory pain.
iv. To determine the effects of α7 nAChR ligand on BDNF expression in the hippocampus in neuroinflammatory pain
v. To examine the effects of α7 nAChR ligand on NKCC1 and KCC2 expression in the hippocampus in neuroinflammatory pain.

Chapter 2: Effects of α7 nAChR PAM on LPS-induced neuroinflammatory pain and microglial activation in the hippocampus in mice

2.1. Introduction

As described in chapter 1, the α7 nAChRs have been implicated in the modulation of pain in animal models and are widely distributed on non-neuronal cells such as microglial cells in CNS, including hippocampus (Dineley et al., 2015; Gaimarri et al., 2007; Vincler, 2005). Consistent with previous studies, cholinergic nicotinic pathway exists in the hippocampus involving microglial α7 nAChRs (Shytle et al., 2004). The cholinergic nicotinic pathway in the hippocampus has been found to regulate neuroinflammation. During neuroinflammation, microglia cells rapidly change from a resting state to an active state and produce variety of mediators, including Iba-1, the microglial activation marker (Yoon et al., 2012). Therefore, increased expression of Iba-1 corresponds to the severity of microglial activation in neuroinflammation. Previous studies showed that microglial activation in the CNS plays a critical role in the onset and maintenance of neuroinflammatory pain (Austin and Moalem-Taylor, 2010; Yoon et al., 2012).

The LPS, an exogenous ligand for TLR4, is widely implicated for microglial activation in the CNS (Silverman et al., 2014). Recent studies indicate that LPS increases nociceptive sensitivity to pain stimuli resulting in allodynia and thermal hyperalgesia likely due to increased microglial activation in the CNS (Yoon et al., 2012). Moreover, the α7 nAChR agonists have been found to reduce neuroinflammatory pain in preclinical studies due to their ability to decrease CNS microglial activation (Loram et al., 2010; Pocock and Kettenmann, 2007). Therefore, α7 nAChR PAM, including TQS might be

better therapeutic candidate for neuroinflammatory pain likely due to its ability in inhibit α7 nAChR desensitization.

Methyllcaconitine (MLA) is a norditerpenoid alkaloid and potent competitive α7 nAChR antagonist (Davies et al., 1999; Holladay et al., 1997; Williams et al., 1994). The MLA possesses high affinity for α7 nAChR (Sharples and Wonnacott, 2001). The MLA has been known to cross BBB after peripheral administration (Turek et al., 1995). It has been indicated to block α7 nAChR-mediated effects in animal models (Callahan et al., 2013; Freitas et al., 2013). Therefore, MLA is used to examine the CNS effects of the α7 nAChR during in vivo studies (Turek et al., 1995).

Given this, the evidence supports the involvement of microglial cells in neuroinflammatory pain involving α7 nAChR in the CNS, The TQS might exert similar effects on hippocampal microglial cells, thus decreasing microglial activation and neuroinflammatory pain. Therefore, we hypothesized that TQS will reduce microglial activation associated pain by reducing LPS-induced microglial activation in the hippocampus. In the present study, we examined the antihyperalgesic and antiallodynic effects of TQS following systemic administration of LPS in mice model. Moreover, we examined the effects of MLA on TQS-induced antiallodynic and antihyperalgesic effects. Additionally, we have determined the effects of TQS on microglial Iba-1 expression in the hippocampus in mice.

2.2. Methods

Animals

All experiments were conducted on male C57BL/6J mice purchased from Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed in groups of four in standard

shoebox cages (29×18×12 cm), under standard laboratory conditions (22 ± 2 °C, relative humidity 50–60%) and maintained on a 12-h light/dark cycle (lights on at 0600 h) with unlimited access to food and water. Mice were 10–12 weeks of age at the beginning of the experiment. The behavioral experiments were carried out between 0900 and 1600 h in a blind manner with respect to drug treatment. All procedures were in compliance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at South Dakota State University. Good Laboratory Practice and ARRIVE guidelines were obeyed. All efforts were attempted to ensure least animal suffering.

Drug treatment

Lyophilized LPS (*Escherichia coli*, serotype 055:B5) and MLA were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were dissolved in normal saline (0.9% NaCl). TQS was purchased from Tocris Bioscience (Ellisville, MO, USA) and was reconstituted in normal saline containing 1% dimethyl sulphoxide (DMSO) and 0.5% tween 80. Mice received TQS (1 or 4 mg/kg) 0.5 h before LPS administration. The MLA was injected 10 min before TQS injection. All drugs were administered intraperitoneally (i.p.) in a volume of 10 ml/kg of body weight.

Induction of tactile allodynia and thermal hyperalgesia

Tactile allodynia and thermal hyperalgesia were induced by LPS (1 mg/kg, i.p.) administered six hours (h) before measurement of tactile allodynia and thermal hyperalgesia as described previously with minor modification (Yoon et al., 2012). Control animals were given an equal volume of vehicle.

Tactile allodynia

A standardized testing method was used to measure tactile allodynia as described previously (Chaplan et al., 1994), six h following LPS administration. Briefly, mice were acclimatized to the wire mesh boxes (Stoelting, Inc., Wood Dale, IL, USA) for 4 successive days prior to testing for tactile allodynia. On the day of testing, mice were permitted to habituate for 30 $\pm$ 5 min. A logarithmic series of calibrated von Frey filaments (Stoelting, Inc., Wood Dale, IL, USA) were to determine 50% paw withdrawal threshold using up-down method of Dixon with minor modification (Dixon, 1980). A series of filaments beginning with one that had 0.16 g buckling weight were applied vertically to right/left hind paw at its plantar surface. Lifting of the right/left hind paw was noted as positive score and the next light force filament was then applied for second measurement. If paw withdrawal did not happen within 5 seconds (s) of application, the next higher filament was applied. This process was continued until four readings were taken after initial positive response or until five successive negative scores (0.6, 1, 1.4, 2; 2.0 g score was assigned) or four consecutive positive measurements (0.16, 0.07, 0.04, 0.02, 0.008; 0.008 g score was assigned). Von Frey filaments were applied at several s of intervals thus leading to resolution of any behavioral response to earlier stimulus. Ambulation was noted as ambiguous response, and if it happened, stimulus was repeated. The resulting chain of positive and negative scores was used to calculate 50% paw withdrawal threshold. 50% g paw withdrawal threshold = $(10^{(Xf + K\delta)})/10000$, Where K= tubular value for pattern of positive and negative responses, Xf = Log value of final von Frey filament force use, and δ=Log value of mean difference between stimuli.

Thermal hyperalgesia

Thermal hyperalgesia was determined six h following LPS administration by measuring changes in hind paw withdrawal latency using a plantar analgesia apparatus (IITC Life Science Inc., Woodland Hills, CA) as described previously (Yoon et al., 2012). Briefly, paw withdrawal (latency time) was determined using an intense heat source to stimulate thermoreceptor in the sole of the foot. Each mouse was subjected to latency on hot plate maintained at 54.0 $\pm$ 0.1^{o}C in a plexiglas chamber. Animals jumping, licking or flicking was noted as positive measurement. Latency for each mouse was noted as a mean of three readings. Thirty s was chosen as a cut-off time to avoid tissue damage.

Western blot analysis

Mice were sacrificed six h later LPS administration, their hippocampi were dissected out, frozen in dry ice, and stored at −80°C until further analysis. Western blot analysis was carried out as described previously with minor modifications (Yoon et al., 2012). Briefly, brain tissue samples were homogenized in RIPA buffer having Dulbecco's Phosphate-buffered saline (Atlanta Biologicals, Lawrenceville, GA, USA), 1% Igepal CA-630 (Sigma-Aldrich, St. Louis, MO, USA), 0.1% sodium dodecyl sulfate (Fisher Scientific, Fair Lawn, New Jersey, USA), and protease inhibitor mix (complete, Mini, Roche, Indianapolis, IN, USA). Each sample was then centrifuged (17,000×g, 20 min at 4°C) and supernatant was collected. Total protein concentration in the hippocampal samples was measured by bicinchoninic acid assay (Pierce, Rockford, IL, USA). Equal amounts of protein (50 μg) were loaded onto 12% gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis using electrophoresis unit (Amersham Biosciences, San Francisco, CA, USA). Separated proteins were then transferred onto nitrocellulose

membranes (Santa Cruz Biotechnology, Dallas, Texas, USA) at 40 V overnight using Mini Tank Transfer Unit (Amersham Biosciences, San Francisco, CA, USA). Membranes were then blocked on a gyro-rocker having 5% nonfat dry milk in tris-buffered saline/0.1% tween-20 for 50 min, and subsequently incubated overnight at 4°C with primary antibodies for Iba-1 (goat anti-rabbit-Iba-1; 1:1000, Wako, Osaka, Japan), or β-tubulin (E7-S, 1:5,000, mouse monoclonal, University of Iowa, USA). After incubation with primary antibody, membranes were incubated with proper horseradish peroxide-conjugated secondary antibodies (Santa Cruz Biotechnology, Dallas, Texas, USA). Bound antibodies were then detected with enhanced chemiluminescence prime reagent (Amersham, Buckinghamshire, UK), and protein quantification was determined using densitometric analysis. Western blot image that was close to average was chosen as representative image.

Statistical analyses

For hyperalgesia, data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons or Student's t-test for MLA data. For tactile allodynia, Kruskal-Wallis test followed by Dunn's post hoc test for multiple comparisons or Mann-Whitney test for MLA data was used. Data from Western blot studies were expressed as Iba-1/β-tubulin expression (% control) and were analyzed by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons using GraphPad Prism 5.0 (GraphPad Inc., San Diego, CA, USA). The difference between treatments was considered significant at $P < 0.05$. All results are expressed as mean ± standard error of mean (S.E.M.).

2.3. Results

Effects of TQS on 50% paw withdrawal threshold during von Frey filament test in LPS-*induced neuroinflammatory pain*

The effects of TQS on 50% paw withdrawal (tactile allodynia) are shown in the Figure 5. The results from Kruskal-Wallis test showed that TQS pretreatment significantly affected 50% paw withdrawal threshold in von Frey filament test ($P < 0.0001$). Dunn's post hoc test for multiple comparison showed that LPS (1 mg/kg) significantly ($P < 0.001$) decreased 50% paw withdrawal threshold as compared to control indicating the presence of tactile allodynia. Moreover, TQS (4 mg/kg) significantly ($P < 0.01$) increased 50% paw withdrawal threshold compared to LPS treatment group indicating significant antiallodynic response.

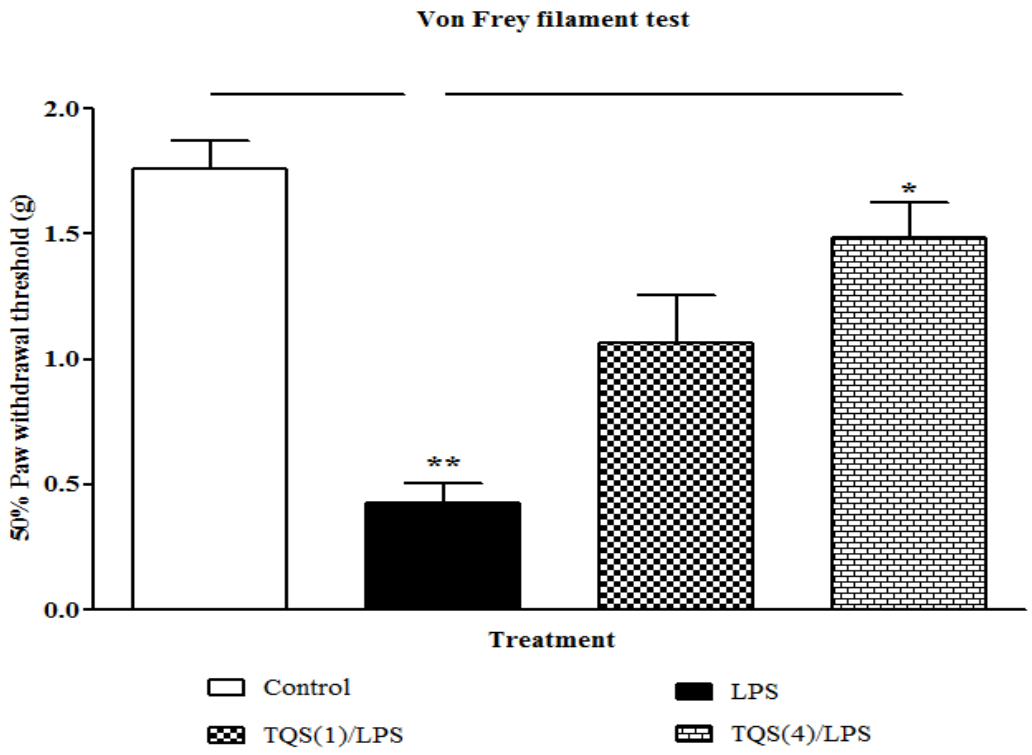

Figure 5: Lipopolysaccharide (LPS) significantly decreased 50% paw withdrawal threshold (tactile allodynia) compared to control. Moreover, TQS significantly increased 50% paw withdrawal threshold compared to LPS treated group. Mice received TQS (1 or 4 mg/kg, i.p.) 0.5 h before the administration of LPS. Control animals received equal volume of vehicle. Paw withdrawal threshold was measured using von Frey filaments six h after LPS administration. Data are expressed as mean ± S.E.M. (n = 7-8/group). *P < 0.01; **P < 0.001.

Effects of TQS on 50% paw withdrawal threshold in von Frey filament test

The effects of TQS on 50% paw withdrawal (tactile allodynia) are shown in the Figure 6. The results from Kruskal-Wallis test showed that TQS did not significantly affect 50% paw withdrawal threshold in von Frey filament test.

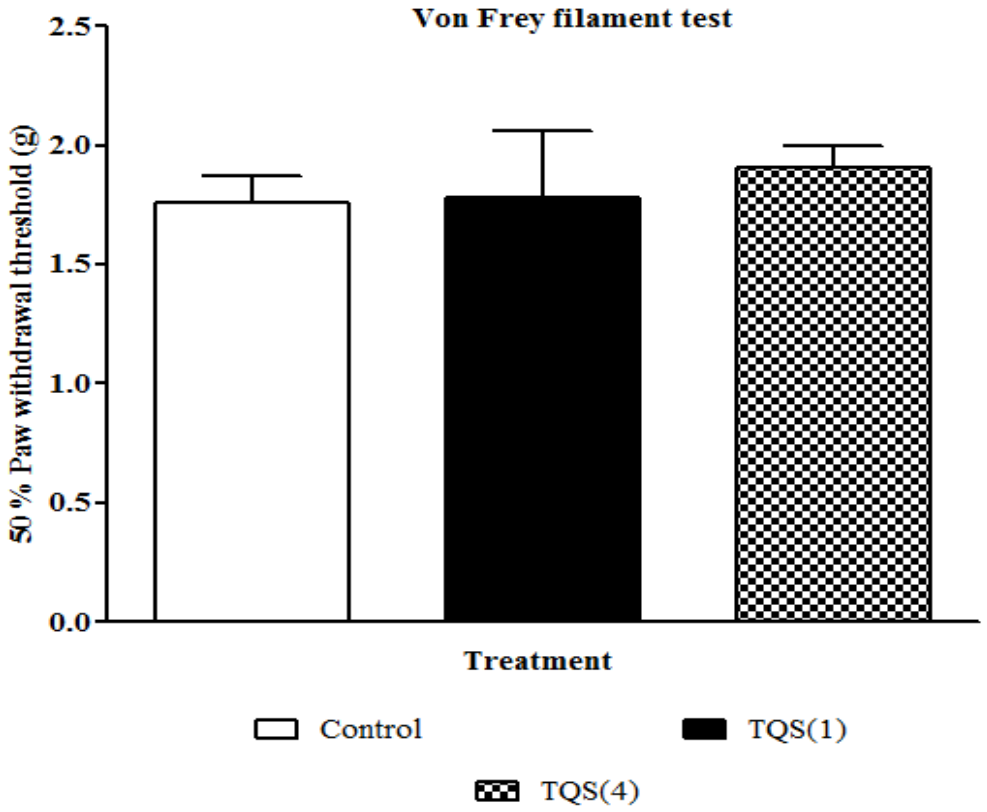

Figure 6: TQS (1 or 4 mg/kg, i.p.) did not significantly affect 50% paw withdrawal (tactile allodynia) in von Frey filament test. Mice received TQS (1 or 4 mg/kg, i.p.) 0.5 h before the administration of saline (Sal). Control animals received equal volume of vehicle. Paw withdrawal threshold was measured using von Frey filaments six h after Sal administration. Data are expressed as mean $\pm$ S.E.M. (n = 4-7/group).

Effects of TQS on latency time during hot plate test in LPS-induced neuroinflammatory pain

The effects of TQS on latency time (thermal hyperalgesia) in hot plate test (54.0 $\pm$ 1^{o}C) are shown in Figure 7. The results from One way ANOVA showed that TQS pretreatment significantly changed latency time in hot plate test ($F_{3,20} = 8.359$; $P < 0.001$). Multiple comparisons of means indicated that LPS (1 mg/kg) significantly ($P < 0.001$) reduced latency time on hot plate as compared to control showing significant reduction in response to heat stimulus. Moreover, TQS (4 mg/kg) significantly ($P < 0.01$) increased latency time on hot plate as compared to LPS treatment group indicating administration of TQS prevented the development of thermal hyperalgesia.

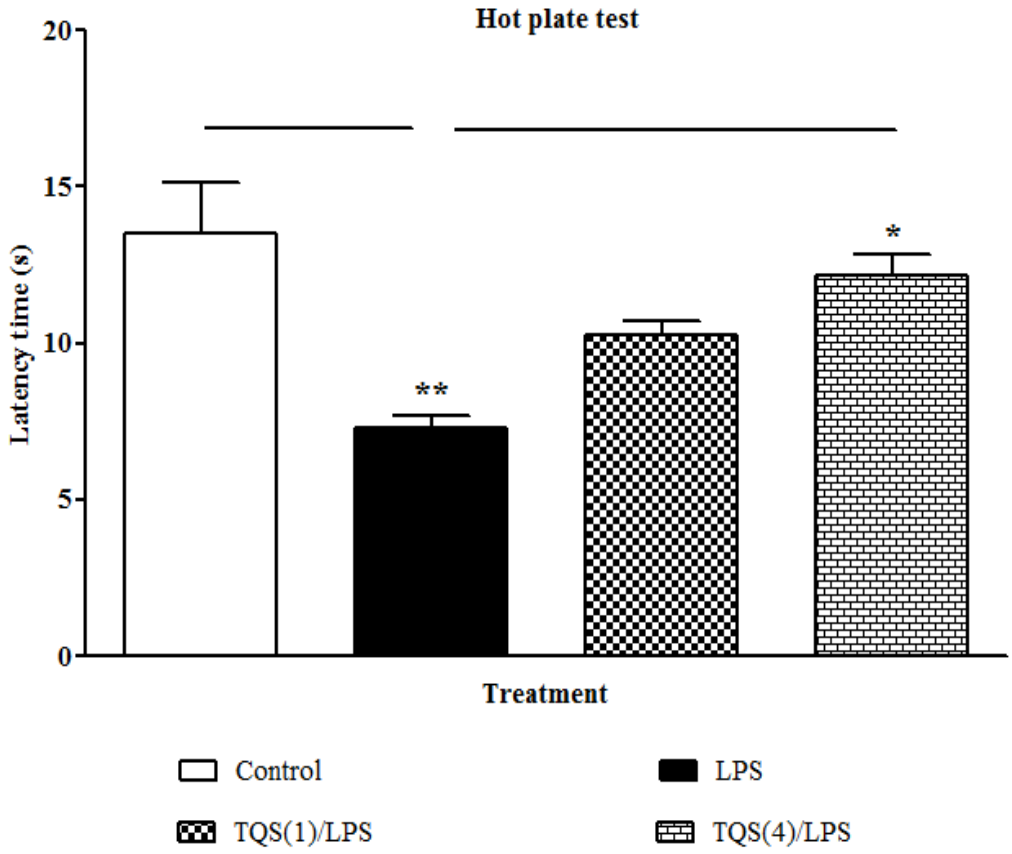

Figure 7: Lipopolysaccharide (LPS) significantly decreased latency time (thermal hyperalgesia) on the hot plate. Moreover, TQS significantly increased latency time on hot plate compared to LPS treated group. Mice received TQS (1 or 4 mg/kg, i.p.) 0.5 h before the administration of LPS. Control animals were given equal volume of vehicle. Latency on hot plate was determined six h after LPS administration. Data are expressed as mean ± S.E.M. (n = 6/group). *$P < 0.01$; **$P < 0.001$.

Effects of TQS on latency time in hot plate test

The effects of TQS on latency time (thermal hyperalgesia) in hot plate test (54.0 ± 1^{o}C) are shown in Figure 8. The results from one way ANOVA showed that TQS did not significantly change latency time in hot plate test ($F_{2,11} = 0.8660$; $P > 0.05$).

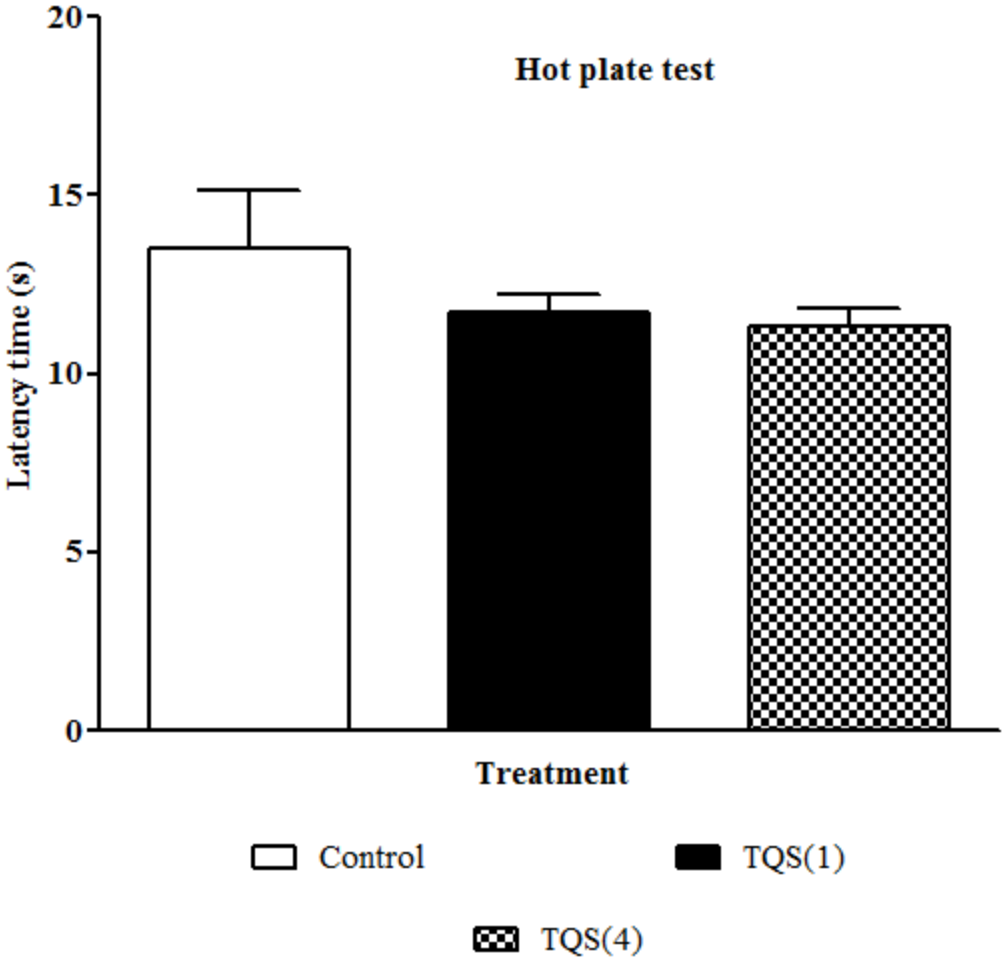

Figure 8: TQS (1 or 4 mg/kg, i.p.) did not significantly change latency time (thermal hyperalgesia) on the hot plate. Mice received TQS (1 or 4 mg/kg, i.p.) 0.5 h before the administration of saline (Sal). Control animals were given equal volume of vehicle. Latency on hot plate was determined six h after Sal administration. Data are expressed as mean ± S.E.M. (n = 4-5/group).

Effects of MLA on TQS-induced antiallodynic response in von Frey filament test

The effects of MLA on TQS-induced antiallodynic response are shown in the Figure 9. The results from Mann-Whitney test showed that MLA (3 mg/kg) pretreatment

significantly ($P < 0.01$) reduced 50% paw withdrawal threshold compared to TQS treatment group, validating that antiallodynic effects of TQS are mediated by α7 nAChR.

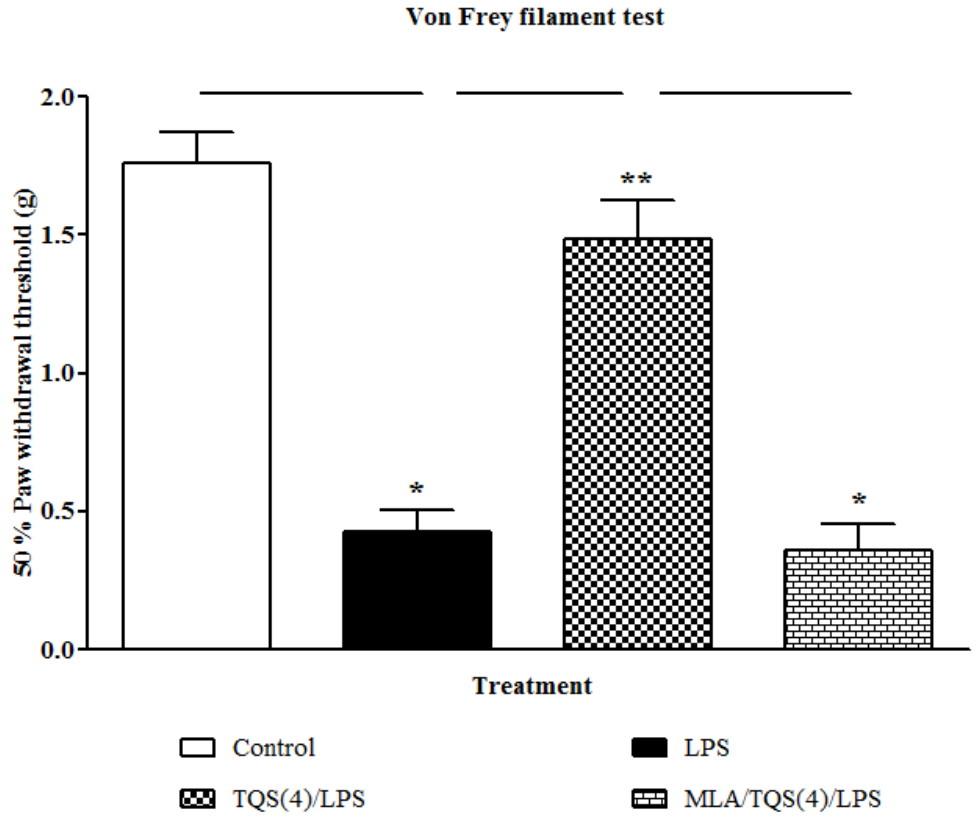

Figure 9: Lipolpolysaccharide (LPS) significantly decreased 50% paw withdrawal threshold (tactile allodynia) compared to control. Moreover, TQS significantly increased 50% paw withdrawal threshold compared to LPS treatment group. Methyllycaconitine (MLA) significantly reduced 50% paw withdrawal threshold compared to TQS treatment group. Mice were given TQS (4 mg/kg, i.p.) 0.5 h before LPS administration. Animals received MLA (3 mg/kg, i.p.) ten min before TQS injection. Control animals received equal volume of vehicle. Paw withdrawal threshold was measured using von Frey filaments six h after LPS administration. Data are expressed as mean ± S.E.M. (n = 6-8/group). *$P < 0.01$; **$P < 0.001$.

Effects of MLA on TQS-induced antihyperalgesic response in hot plate test

The effects of MLA on TQS-induced antihyperalgesic response are shown in the Figure 10. Results from Student's t-test showed that MLA (3 mg/kg) pretreatment significantly ($P < 0.001$) decreased latency time on hot plate as compared to TQS treatment group, indicating that MLA significantly reduced antihyperalgesic effects of TQS mediated through α7 nAChR. This validated the involvement of α7 nAChR in the mediation of antihyperalgesic effects of TQS.

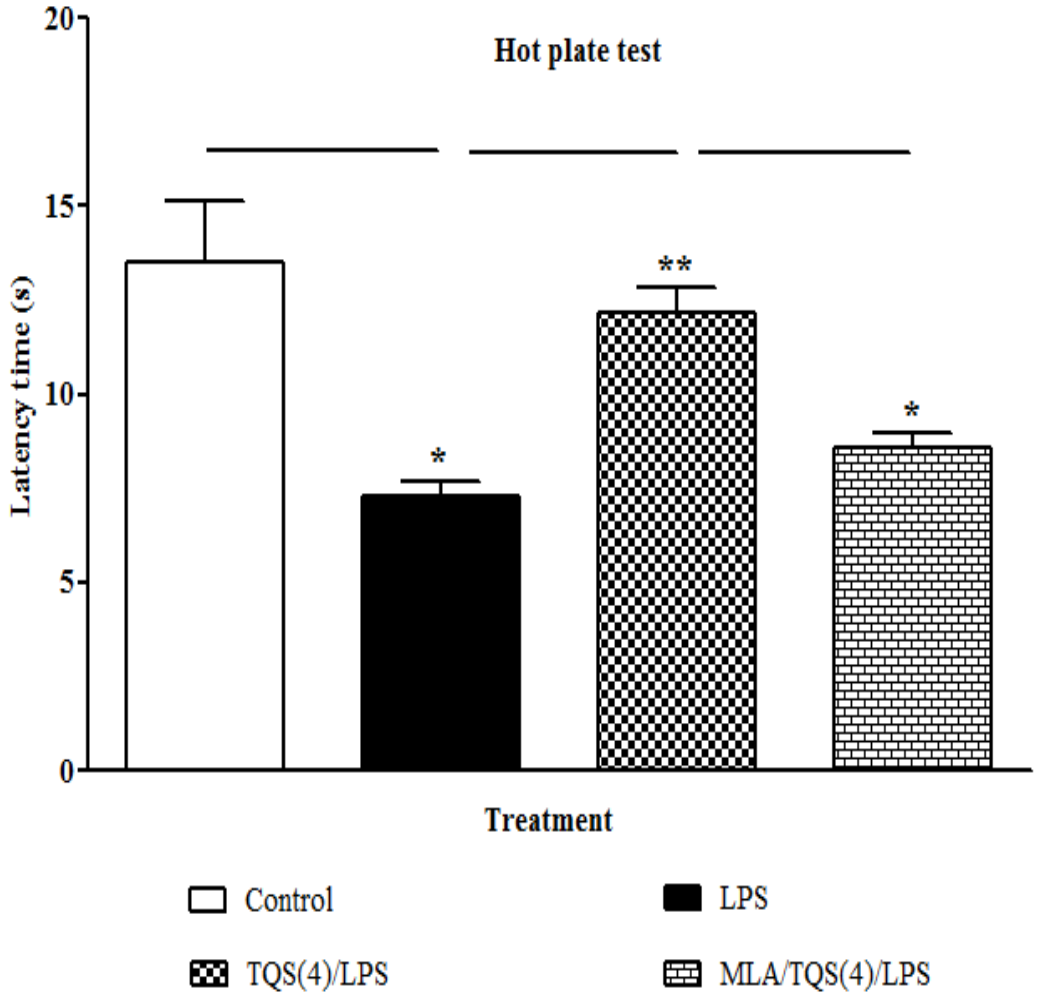

Figure 10: Lipolpolysaccharide (LPS) significantly decreased 50% latency time (thermal hyperalgesia) on hot plate compared to control. Moreover, TQS significantly increased latency time on hot plate compared to LPS treatment group. Methyllycaconitine (MLA) significantly reduced latency time on the compared to TQS treatment group. Mice received TQS (4 mg/kg, i.p.) 0.5 h before LPS administration. The MLA (3 mg/kg, i.p.) was administered 10 min before TQS injection. Control animals received equal volume of vehicle. Latency on hot plate was measured six h after LPS administration. Data are expressed as mean $\pm$ S.E.M. (n = 6/group). *$P < 0.01$; **$P < 0.001$.

Effects of TQS on LPS-induced increased Iba-1 expression in hippocampus

The effects of TQS on Iba-1 exprssion in the hippocampus are shown in the Figure 11. The results from one way ANOVA showed that TQS pretreatment significantly affected Iba-1 expression in the hippocampus ($F_{3,23} = 12.90$; $P < 0.0001$). Multiple comparisons of means showed that LPS (1 mg/kg) significantly ($P < 0.01$) increased the expression of Iba-1 in the hippocampus as compared to control indicating that administration of LPS activated microglial cells in the hippocampus. Additionally, TQS (1 or 4 mg/kg) significantly ($P < 0.01$ or $P < 0.001$) reduced Iba-1 expression in the hippocampus as compared to LPS treatment group. This showed that antiallodynic and antihyperalgesic effects of TQS are mediated through the inhibition of microglial activation in the hippocampus.

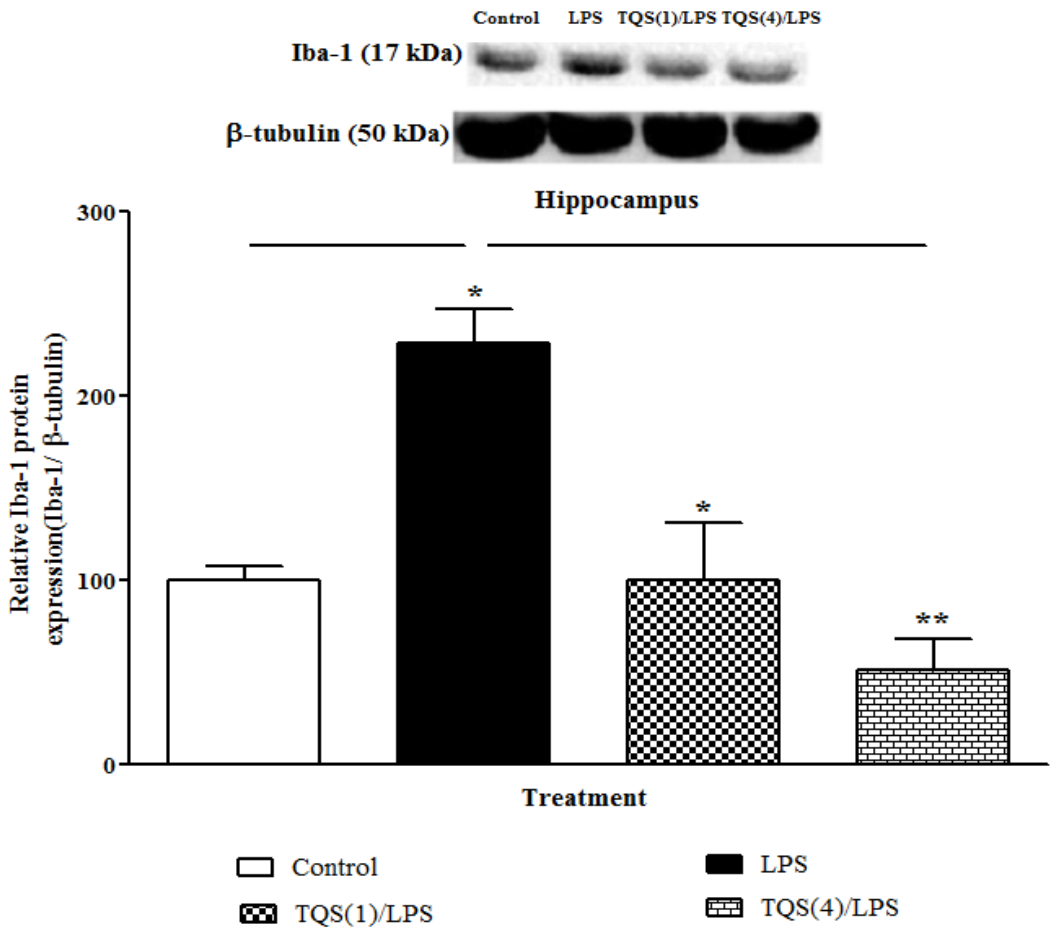

Figure 11: Lipolpolysaccharide (LPS) significantly increased the expression of ionized calcium-binding adapter molecule 1 (Iba-1) compared to control in the hippocampus. Furthermore, TQS (1 or 4 mg/kg, i.p.) significantly reduced Iba-1 expression in the hippocampus compared to LPS treated group. Animals received TQS (1 or 4 mg/kg, i.p.) 0.5 h before LPS administration. Control animals received equal volume of vehicle. Hippocampi were collected 6 h after LPS administration. Representative Western blots for Iba-1 expression from hippocampus (top panel). Data are expressed as mean $\pm$ S.E.M. (n = 6-7/group). *$P < 0.01$; **$P < 0.001$.

2.4. Discussion

The data from current study demonstrated the effectiveness of TQS in LPS-induced neuroinflammatory pain. Our findings indicate that pretreatment of TQS, an α7 nAChR PAM, dose-dependently produced antiallodynic and antihyperalgesic effects in LPS-induced neuroinflammatory pain model. These analgesic effects were reversed by MLA, an antagonist at α7 nAChR, indicating the involvement of α7 nAChR. In addition, pretreatment of TQS significantly reduced the LPS-induced increase in hippocampal Iba-1, a microglial activation marker, expression suggestive the inhibition of hippocampal microglial activation resulted in reversal of tactile allodynia and thermal hyperalgesia in our mice model.

Previous studies have indicated that α7 nAChR agonists reduce pain hypersensitivity in animal models. For example, α7 nAChR selective agonists effectively decrease neuropathic and neuroinflammatory pain-like symptoms in animal models (Feuerbach et al., 2009; Loram et al., 2010; Loram et al., 2012). Choline, an α7 nAChR agonist and metabolite of ACh, was found to reduce in postoperative pain in animal models (Rowley et al., 2010; Rowley et al., 2008). Additionally, i.c.v. injection of citicoline, precursor for choline, was effective in inflammatory pain models (Bagdas et al., 2011). However, as reported earlier, α7 nAChRs become rapidly desensitized in the presence of agonist (Papke et al., 2009). One alternative approach is to selectively increase the activity of α7 nAChRs agonists at α7 nAChRs is through PAMs (Freitas et al., 2013; Munro et al., 2012; Williams et al., 2011).

Previous studies have shown that PAMs facilitate endogenous neurotransmission and increase the efficacy and potency of an agonist without directly stimulating the agonist

binding site (Williams et al., 2011). In principle, the α7 nAChRs PAMs do not show intrinsic activity at the receptor, however, they enhance endogenous cholinergic neurotransmission (Freitas et al., 2013). These α7 nAChRs PAMs have been classified as either type I, such as NS1738, or type II, such as TQS, based on difference in their effects on receptor desensitization (Collins et al., 2011). Type I PAMs have negligible effect on desensitization kinetics, whereas type II PAMs can efficaciously prevent desensitization and reactivate already desensitized α7 nAChRs (Gronlien et al., 2007). Administration of α7 nAChRs type II PAM is associated with decreased pain sensitivity in animal models (Alsharari et al., 2013; Freitas et al., 2013; Munro et al., 2012; Pacini et al., 2010).

The data presented in this study indicated for the first time that pretreatment of TQS reduced both thermal hyperalgesia and tactile allodynia in our mouse model. Taken together, these data support our findings regarding the effectiveness of TQS in inhibiting tactile allodynia and thermal hyperalgesia induced by LPS. Our results also demonstrate that antiallodynic and antihyperalgesic effects of TQS are linked with α7 nAChRs activation since prior treatment with MLA completely reversed the effects produced by TQS. To the best of our knowledge, we report for the first time that in neuroinflammatory pain model, TQS blocked tactile allodynia and thermal hyperalgesia in an a α7 nAChR specific manner. Therefore, these results strongly support the involvement of α7 nAChRs positive allosteric modulation in pain transmission in the CNS.

Although microglial cells are considered as scavenger cells in the CNS, emerging evidence indicates that microglial cells actively communicate with neurons and substantially contribute to the pathophysiology of various types of neurodegenerative disorders such as neuroinflammatory pain (Carnevale et al., 2007). Activation of

microglial cells is now known as being fundamentally important in the induction and maintenance of pathological pain conditions in animal models involving α7 nAChRs in the CNS (Loram et al., 2010; Medhurst et al., 2008). Microglial cells experience rapid activation in the CNS due to pathological events, and provide a strong force for pathological pain conditions such as neuroinflammatory pain (Yoon et al., 2012; Zhuo et al., 2011). For example, peripheral nerve injury mediates increased expression of microglial activation marker, including Iba-1 (Garrison et al., 1991). Additionally, studies have indicated that microglial activation is adequate to induce pain hypersensitivity in animal models (Tsuda et al., 2003). Therefore, reducing microglial activation may have potential therapeutic utility for painful sensation and/or neurological disorders.

The clinical potential of targeting activated microglial cells is now becoming highly recognized but is still in its infancy stage due to identification of optimal drug targets for controlling neuroinflammatory pain associated with microglial activation (Wen et al., 2011; Zhuo et al., 2011). For example, microglial inhibitors such propentofylline reduce pain hypersensitivity (Sweitzer et al., 2001). Conversely, fractalkine, the microglial activator, results in development of behavioral signs of neuroinflammatory pain, including tactile allodynia, and thermal hyperalgesia (Milligan et al., 2004).

Previous studies showed that systemic injection of LPS resulted in microglial activation due to increased morphological changes associated with increased Iba-1 expression in the hippocampus compared to most of other brain regions (Silverman et al., 2014). Moreover, microglial cells were found to be densely populated in the hippocampus as compared to other brain regions, including brainstem (Lawson et al.,

1990). Additionally, α7 nAChRs are expressed at higher density in the hippocampus than in most of the CNS regions (Gaimarri et al., 2007; Gotti et al., 2006; Picciotto et al., 2000) indicating TQS might exert α7 nAChR-mediated effects more in hippocampus than in other brain regions. Furthermore, cholinergic nicotinic pathway has been proposed to exist in the hippocampus that plays very important role in the inhibition of microglial activation involving α7 nAChRs (Shytle et al., 2004). Thus, decreasing microglial activation in the hippocampus through α7 nAChRs may have therapeutic potential for neuroinflammatory pain.

In line with these reports, we noted that LPS increases the Iba-1 expression in the hippocampus in our neuroinflammatory pain model. Clearly, these findings indicate that there might be a correlation between hippocampal microglial activation and neuroinflammatory pain following LPS administration. We have also found that TQS reduces the activation of microglial cells in the hippocampus as indicated by reduced Iba-1 expression during antiallodynic and antihyperalgesic response. This observation also supports the role of microglial cells in the active modulation of neuroinflammatory pain-like symptoms. Taken together, we propose that antiallodynic and antihyperalgesic effects of TQS are likely due to reduced hippocampal microglial activation involving α7 nAChRs.

The underlying mechanisms involved in the antiallodynic and antihyperalgesic effects of TQS in neuroinflammatory pain models are currently not known. Previous studies have demonstrated that NF-κB becomes activated during microglial activation (Loram et al., 2010; Uesugi et al., 2006) and NF-κB stimulated gene expression contributes to the development of increased excitability of nociceptive neurons. Hence, NF-κB inhibition in

the hippocampus is possibly important tool for the significant antiallodynic and antihyperalgesic effects of TQS. Therefore, subsequent studies are required to examine how TQS affects hippocampal microglial cells beyond the expression of activation marker, including the quantification of NF-κB expression. It is noteworthy to mention that TQS could increase endogenous cholinergic transmission through α7 nAChRs in the hippocampus, reducing pain-related behavior involving inhibition of NF-κB activation. The central effects of TQS may widen its potential for clinical usefulness for neuroinflammatory pain as neuroinflammatory pain-like symptoms are possibly mediated by activated microglial cells. Therefore, the results from this study indicate that α7 nAChR-dependent mechanisms are critically involved in the modulation of neuroinflammatory pain-like symptoms in this mouse model.

In conclusion, our data indicate that antiallodynic and antihyperalgesic effects produced by TQS might be due to positive allosteric modulation of α7 nAChRs on microglial cells likely in the hippocampus. The evidence further supports the concept that α7 nAChRs PAM might represent novel and attractive therapeutic target for treating referred tactile allodynia and thermal hyperalgesia associated with microglial activation in neuroinflammatory pain.

Chapter 3: Effects of α7 nAChR PAM on NF-κB activation in the hippocampus

3.1. Introduction

We have demonstrated that TQS reduces neuroinflammatory pain by decreasing microglial activation in the hippocampus (see chapter 2). It was shown that NF-κB increases in the hippocampus in painful conditions (Chou et al., 2011). Moreover, previous studies showed that microglial α7 nAChRs activation causes increased NF-κB inactivation in neuroinflammatory pain involving enhanced expression of IκB mRNA (Egea et al., 2015; Loram et al., 2010). However, as reported earlier, these receptors undergo rapid desensitization upon agonist binding at orthosteric site (Papke et al., 2009), and adopt non-conducting conformation (Williams et al., 2011). The α7 nAChR type II PAMs which prevent α7 nAChR desensitization are known to reduce pain hypersensitivity in animal models at spinal level (Bagdas et al., 2016; Freitas et al., 2013; Munro et al., 2012). However, the antialloydynic and antihyperalgesic effects of α7 nAChR type II PAMs in the hippocampus are not examined yet.

The evidence suggests that hippocampus is critically involved in pain production and maintenance and involves dentate gyrus (DG) (Soleimannejad et al., 2007), and CA1 (Khanna and Sinclair, 1989; Martuscello et al., 2012) regions. Compared to other brain regions, DG and CA1 regions of hippocampus are highly populated with microglial cells (Lawson et al., 1990), indicating a potential role of hippocampal microglia in neuroinflammatory pain. Furthermore, evidence suggests that activated NF-κB within microglial cells are important regulators in neuroinflammatory pain development (Loram et al., 2010; Watkins et al., 2001; Zhuo et al., 2011). Telmisartan (TEL), was found to exhibit activity in neuropathic pain (Jaggi and Singh, 2011) in animal models.

Furthermore, TEL was reported to reduce LPS-induced NF-κB activation within macrophages (Balaji and Ramanathan, 2014; Saavedra, 2012). Therefore, NF-κB has emerged as potential target of great importance due to its dysregulation in neuroinflammatory pain conditions (Loram et al., 2010). Therefore, targeting activated NF-κB within hippocampal microglia through α7 nAChRs positive allosteric modulation might have therapeutic utility for neuroinflammatory pain.

Emerging evidence suggests that microglial cells play a very important role in the maintenance of neuroinflammatory pain state involving activated NF-κB and enhanced CD11b expression, a microglial activation marker in the CNS (Loram et al., 2010; Tsuda et al., 2005; Watkins et al., 2001). Previous studies showed that increased expression of IκB mRNA parallels enhanced NF-κB activity and is used as a marker for increased NF-κB activation in neuroinflammatory pain (Ledeboer et al., 2005; Loram et al., 2010; Yoshikawa et al., 2006). Moreover, studies have indicated the involvement of activated NF-κB and increased IκB expression in the mediation of neuroinflammatory pain during microglial activation in the CNS. This amplifies and facilitates pain transmission (Ledeboer et al., 2005; Loram et al., 2010). Furthermore, enhanced expression of CD11b represents the severity of microglial activation during neuroinflammation (Gonzalez-Scarano and Baltuch, 1999; Ling and Wong, 1993; Rock et al., 2004).

Given the previous findings, it is expected that reducing microglial and NF-κB activation in the hippocampus might be of potential therapeutic utility for decreasing neuroinflammatory pain-like symptoms involving α7 nAChR. Therefore, we hypothesized that TQS will decrease microglial NF-κB activation in the hippocampus during antiallodynic and antihyperalgesic effects as reported before (Abbas and Rahman,

2016). In this study, we have determined the effects of TQS and TEL, an inhibitor of NF-kB (Balaji and Ramanathan, 2014; Saavedra, 2012), on IκB mRNA and CD11b mRNA expression in the hippocampus. In addition, we have examined the effects of TQS on p-NF-κB p^{65} expression in the hippocampus in mice.

3.2. Methods

Animals

Male C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). The animals were housed under standard laboratory conditions as described previously (see chapter 2, section 2.2).

Drug treatment

TEL was obtained from Tocris Bioscience (Ellisville, MO, USA) and was reconstituted in normal saline containing 2.6% tween 80 and 2.6% DMSO. The source, method of reconstitution, and time of administration for other drugs used in this study are mentioned previously (see chapter 2, section 2.2). TEL was given 5 min before TQS administration. Neuroinflammatory pain was induced by LPS by the method mentioned earlier (see chapter 2, section 2.2). The doses for TQS, TEL, and MLA were selected based on our previous report and others (Abbas and Rahman, 2016; Haraguchi et al., 2010; Jaggi and Singh, 2011).

Tactile allodynia

Tactile allodynia was measured as described previously (see chapter 2, section 2.2).

Thermal hyperalgesia

Thermal hyperalgesia was quantified as described earlier (see chapter 2, section 2.2).

RNA isolation and complementary (c)*DNA synthesis*

Total RNA from hippocampal tissue was isolated and cDNA was synthesized as mentioned previously with minor modifications (Loram et al., 2010). Briefly, mice were sacrificed through rapid decapitation six h after LPS administration and their hippocampi were dissected out. The tissue sections were then frozen on dry ice, and stored in -80^{o}C refrigerator until further analysis was performed. Hippocampal tissues were then homogenized in the presence of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Chloroform was added to supernatant and total RNA was precipitated from the aqueous phase using isopropyl alcohol. The RNA pellet was then reconstituted in RNAse-free water. The total RNA concentration was determined in a NanoDrop 1000 spectrophotometer (Thermo Scientific, Welmington, DE, USA) at 260 nm. The RNA samples were then stored at -80°C until further analysis. The RNA quality was measured with the OD 260/280 ratio that was between 1.9 and 2.1. The RNA integrity was measured using agarose gel electrophoresis (FisherBiotech electrophoresis system, Mini-Horizontal Unit, Fisher Scientific, Waltham, MA USA). Using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA), the total RNA was then reverse transcribed into first-strand cDNA in Master Cycler Personal (Eppendorf, Hauppauge, NY, USA). The reaction mixture comprised of deoxynucleoside triphosphates Mix, reverse transcription random hexamer primer, reverse transcription buffer, multiscribe reverse transcriptase, RNase inhibitor, and nuclease free water to a

total volume of 20 µl. The mixture was then incubated at 25°C for 10 min, at 37°C for 2 h, and at 85°C for 5 min for enzyme deactivation. All cDNA was stored at -80°C until quantitative real-time polymerase chain reaction (PCR) was performed.

Quantitative real-time PCR

Quantitative real-time PCR was performed as described earlier with minor modification (Loram et al., 2010). Briefly, cDNA template was used for quantitative real-time PCR reaction. Prime primers were used in this study. Their sequences were obtained from Integrated DNA Technologies (Coralville, Iowa, USA) and are displayed in Table 1. Amplification of cDNA was carried out using SYBR Select PCR Master Mix Kit (Applied Biosystems by life technologies, Austin, TX, USA) in StepOnePlus quantitative real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). The reaction mixture (20 ul) consisted of cDNA template, SYBR Select PCR Master Mix, 200 nM of prime primer, nuclease free water. The glyceraldehydes-3-phosphate dehydrogenase (GAPDH) primer was used as housekeeping gene. No multiscribe reverse transcriptase and No RNA controls were used as negative control. Reactions were conducted using 96 wells qPCR plate (Life Technologies, Grand Island, NY, USA). Each sample was used in triplicate and mean cycle threshold (Ct) was calculated for analysis. Cycling parameters were given as; 50°C for 2 min once; 95°C for 2 min once; then 95°C for 15 s, 60°C for 30 s, and 72°C for 1 min and 30 s for 40 cycles ending with a melting curve analysis to control amplification. The relative expression of each gene was determined using delta-delta Ct method. The level of the target mRNA was measured relative to GAPDH and presented as percentage of vehicle control. The expression level of GAPDH was not significantly different between treatments.

Table 1: Prime primer sequence for GAPDH, IκB, and CD11b

Gene	Primer sequence (5'-3')
GAPDH	GTGGAGTCATACTGGAACATGTAG (forward)
	AATGGTGAAGGTCGGTGTG (reverse)
IκB	ATGACAGCTACATGGAGTGG (forward)
	CCAACCCAGAGAACGAAGAG (reverse)
CD11b	TGTCCAGATTGAAGCCATGA (forward)
	CCACAGTTCACACTTCTTTCAG (reverse)

Immunofluorescence assay

Immunofluorescence assay was performed as previously described with minor modification (Ali et al., 2015). Briefly, mice were sacrificed through rapid decapitation six h after LPS administration. Their whole brains were then harvested and fixed with 4% paraformaldehyde for 24 h at room temperature. Mice brains were then cryoprotected with 30% sucrose for 24 h/until brain sank at 4°C and 14 μm coronal sections were cut using Leica cryostat and were fixed on superfrost Plus microscope slides (Fisher Scientific, Pittsburg, PA, USA). Slides were washed with 0.01 molar (M) phosphate-buffered saline (PBS) twice for 5 minute each and were then placed in water bath maintained at 90°C containing 0.01 M citrate buffer (pH 6.0) for 10 min. Hippocampal tissue sections were then blocked in 5% normal bovine serum for 2 h at room temperature having 0.3% triton X-100 in 0.01 M PBS. Tissue sections were incubated with primary antibody for p-NF-κB p^{65} (S536, monoclonal, 1:100, Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. Tissue sections were then incubated with secondary antibody labeled with fluorescein isothiocyanate (FITC) (1:50) (Santa Cruz

Biotechnology, Dallas, TX, USA). The slides were mounted with mounting medium having 4',6'-diamidino-2-phenylindole (DAPI) for nuclear staining and prolong anti-fade reagent (Santa Cruz Biotechnology, Dallas, TX, USA). A negative control lacking primary antibody and treated with secondary antibody was used. Immunofluorescence of representative images was examined using a laser scanning confocal microscope (Olympus Fluoview FV1200). Three tissue sections per animal were used for immunofluorescence assay while two images were captured per each tissue section and all were averaged together. The image that was close to average of all was chosen as representative image. For quantitative analysis, Image J software was used to determine integrated density.

Statistical analyses

For thermal hyperalgesia, immunofluorescence assay, and quantitative real-time PCR, data were analyzed by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. For quantitative real-time PCR for MLA and TQS/TEL combination data for thermal hyperalgesia, Student's t-test was used. For tactile allodynia, Kruskil-Wallis test followed by Dunn's post hoc test for multiple comparisons or Mann-Whitney test for TQS/TEL combination was applied using GraphPad Prism 5.0 (GraphPad Inc., San Diego, CA, USA). The difference between treatments was considered as significant at $P < 0.05$. All results are expressed as mean ± S.E.M.

3.3. Results

Effects of TEL on 50% paw withdrawal during von Fey filament test in LPS-induced neuroinflammatory pain

The effects of TEL on 50% paw withdrawal (tactile allodynia) in von Frey filament test are shown in Figure 12. Kruskal-wallis test showed that TEL significantly decreased 50% paw withdrawal threshold ($P < 0.0001$). Multiple comparisons of means revealed that LPS (1 mg/kg) significantly ($P < 0.001$) decreased 50% paw withdrawal threshold compared to control showing the presence of pain hypersensitivity. Furthermore, TEL (3 mg/kg) significantly ($P < 0.01$) enhanced 50% paw withdrawal threshold compared to LPS treatment group indicating significant antiallodynic effects.

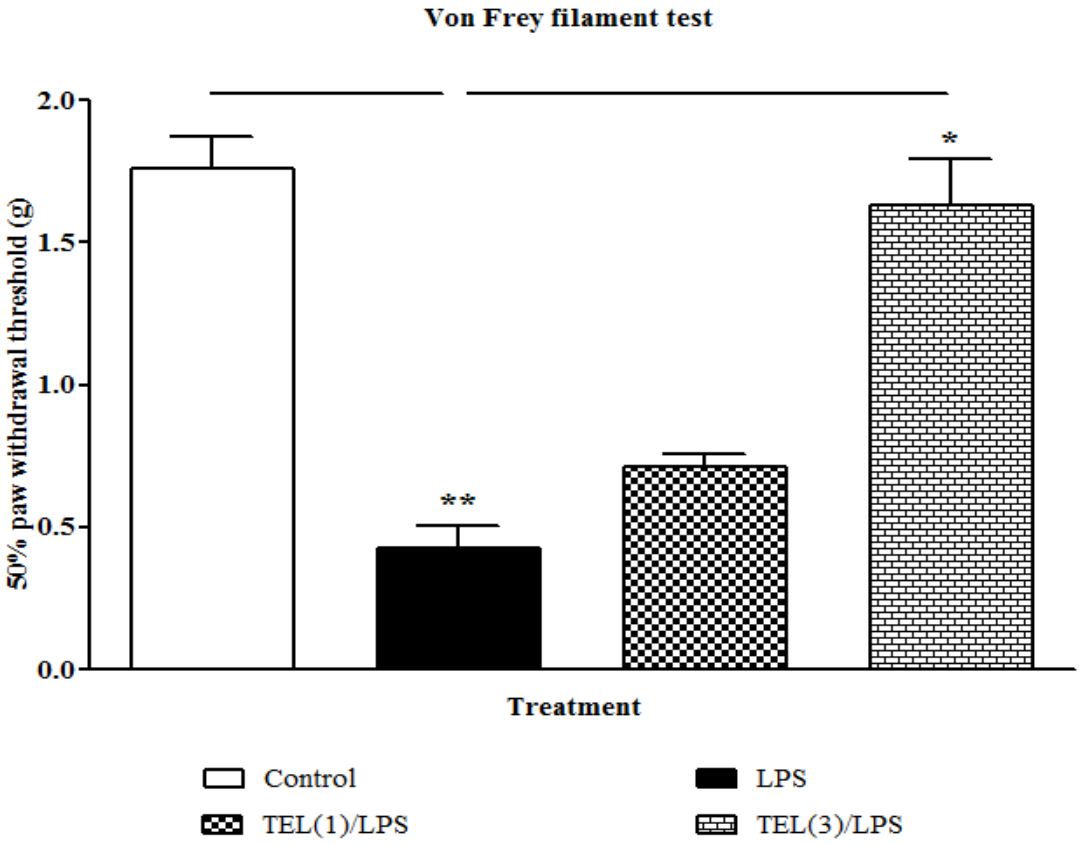

Figure 12: Lipopolysaccharide (LPS) significantly reduced 50% paw withdrawal threshold (tactile allodynia) compared to control. Moreover, telmisartan (TEL) significantly increased 50% paw withdrawal threshold compared to LPS treated group. Mice were administered TEL (1 or 3 mg/kg, i.p.) 0.5 h before LPS administration. Control animals received equal volume of vehicle. Paw withdrawal threshold was determiend using von Frey filaments six h after LPS administration. Data are expressed as mean $\pm$ S.E.M. (n = 6-8/group) $^{*}P < 0.01$; $^{**}P < 0.001$.

Effects of TEL on latency time during hot plate test in LPS-induced neuroinflammatory pain

The effects of TEL on latency time (thermal hyperalgesia) in hot plate test are shown in Figure 13. One way ANOVA showed that TEL significantly altered latency time in hot plate test ($F_{3,20} = 10.52$; $P < 0.001$). Multiple comparisons of means showed that LPS (1 mg/kg) significantly ($P < 0.001$) reduced hot plate latency as compared to control group indicating significant reduction in response to heat stimulus. Moreover, TEL (3 mg/kg) significantly ($P < 0.01$) enhanced hot plate latency compared to LPS treatment group indicating antihyperalgesic effects of TEL are due to the NF-κB inactivation.

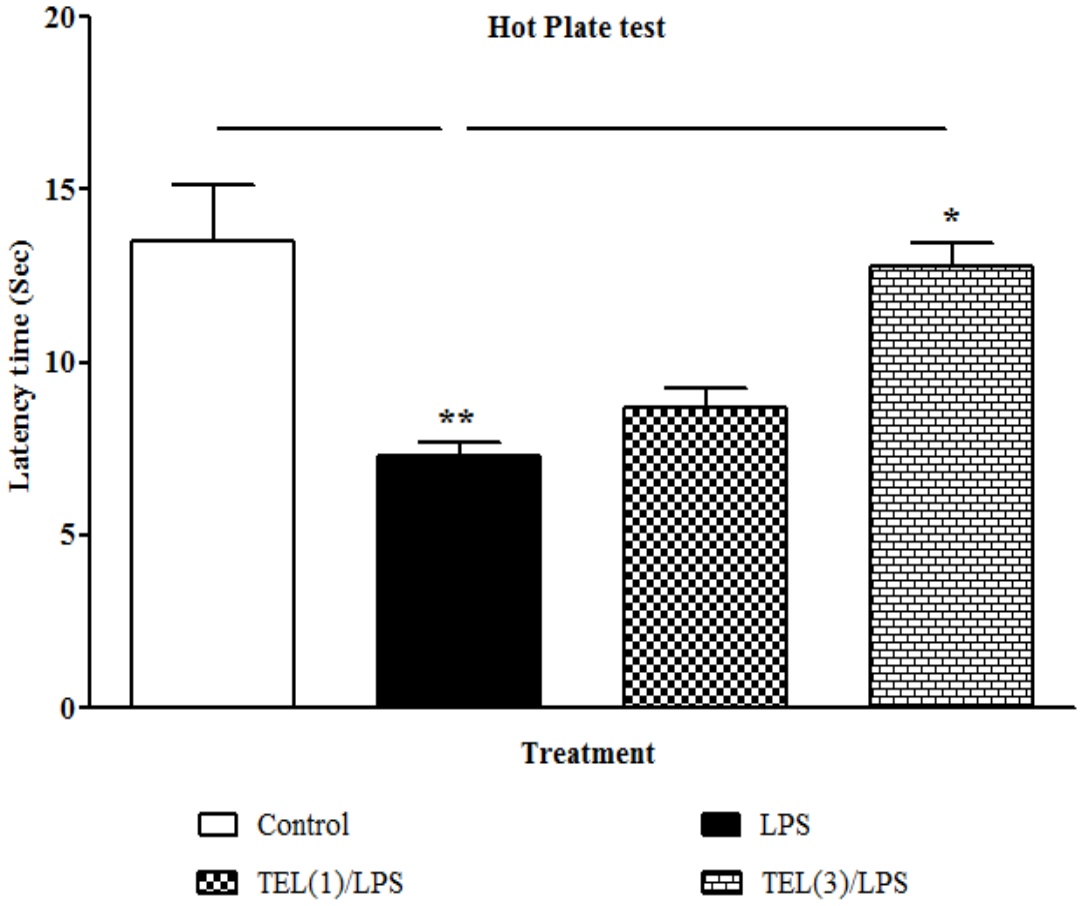

Figure 13: Lipopolysaccharide (LPS) significantly reduced latency time (thermal hyperalgesia) on the hot plate. Moreover, telmisartan (TEL) significantly increased latency time on hot plate compared to LPS treated group. Mice received TEL (1 or 3 mg/kg, i.p.) 0.5 min before the administration of LPS. Control animals received equal volume of vehicle. Latency on hot plate was assessed six h after LPS administration Data are expressed as mean ± S.E.M. (n = 6/group) *$P < 0.01$; **$P < 0.001$.

Effects of co-administration of TQS and TEL on 50% paw withdrawal during von Fey filament test in LPS-induced neuroinflammatory pain

The effects of TQS and TEL co-administration on 50% paw withdrawal (tactile allodynia) in von Frey filament test are shown in Figure 14. Mann-Whitney test showed that co-administration of sub maximal dose of TQS and TEL significantly ($P < 0.05$) decreased 50% paw withdrawal threshold showing decreased activation of NF-κB in TQS-induced antiallodynic effects.

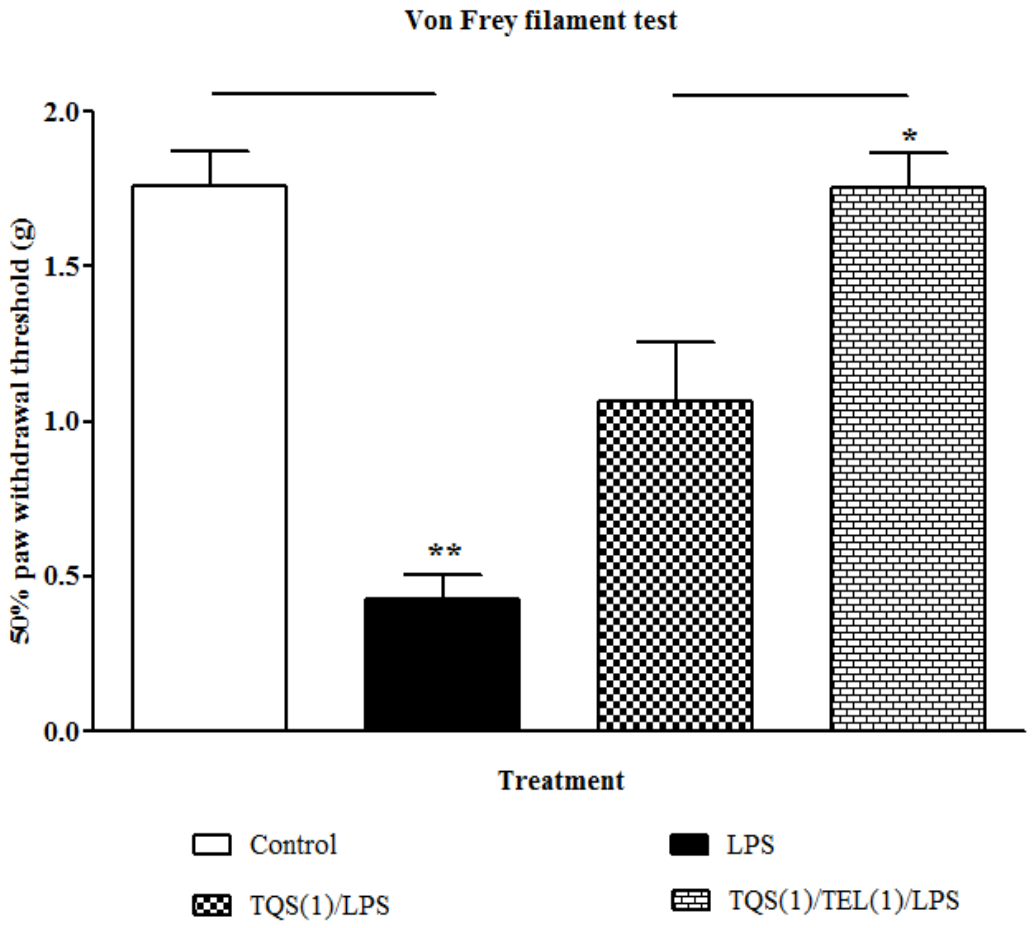

Figure 14: Lipopolysaccharide (LPS) significantly decreased 50% paw withdrawal threshold (tactile allodynia) compared to control. Furthermore, TQS in combination with telmisartan (TEL) significantly increased 50% paw withdrawal threshold compared to TQS treated group. Mice received TEL (1 mg/kg, i.p.) 5 min before TQS (1 mg/kg, i.p.) administration. LPS was administered 0.5 h after TQS administration. Control animals received equal volume of vehicle. Paw withdrawal threshold was measured using von Frey filaments six h after LPS administration. Data are expressed as mean $\pm$ S.E.M. (n = 6-8/group) $^{*}P < 0.05$; $^{**}P < 0.001$.

Effects of co-administration of TQS and TEL on latency time during hot plate test in LPS-induced neuroinflammatory pain

The effects of co-administration of TQS and TEL on latency time (thermal hyperalgesia) in hot plate test are shown in Figure 15. Student's t-test showed that co-administration of sub maximal dose of TQS and TEL significantly ($P < 0.001$) enhanced hot plate latency time showing antihyperalgesic effects of TQS are due to reduced NF-κB activation.

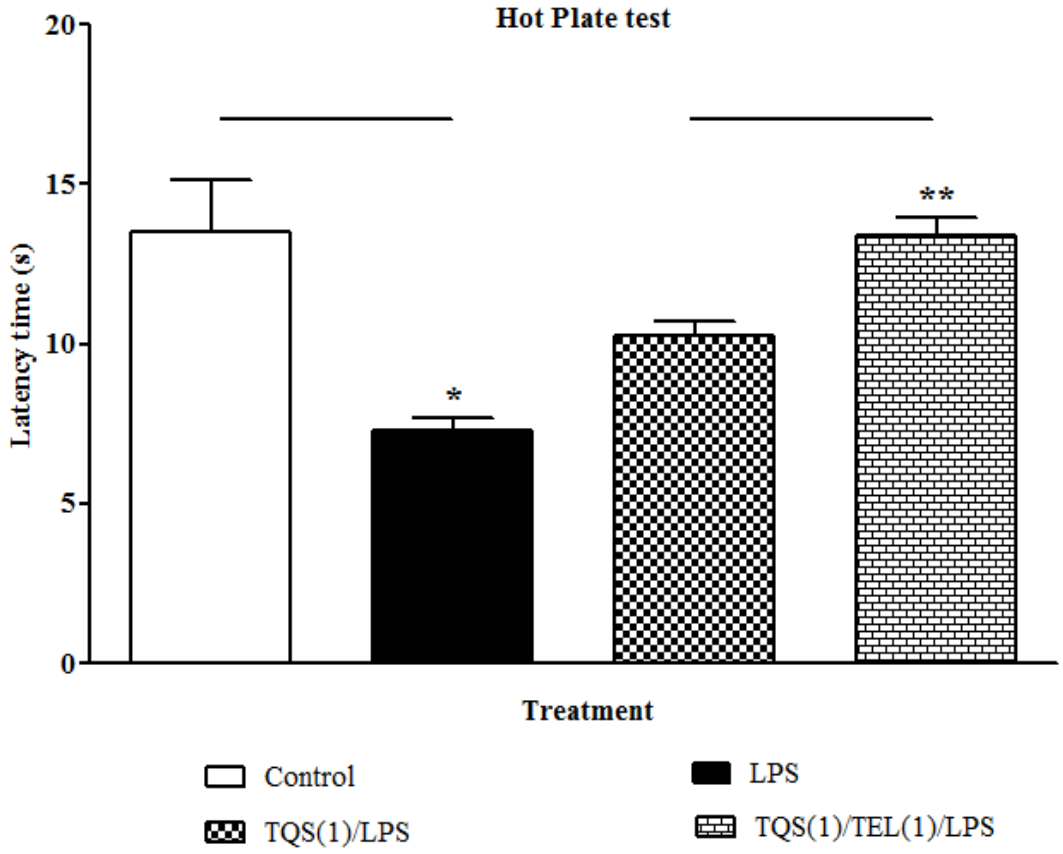

Figure 15: Lipopolysaccharide (LPS) significantly decreased latency time (thermal hyperalgesia) on the hot plate. Moreover, TQS in combination with telmisartan (TEL) significantly increased latency time on hot plate compared to TQS treated group. Mice received TEL (1 mg/kg, i.p.) 5 min before TQS (1 mg/kg, i.p.) administration. LPS was administered 0.5 h after TQS administration. Control animals received equal volume of vehicle. Latency on hot plate was determined six h after LPS administration. Data are expressed as mean ± S.E.M. (n = 6/group). *$P < 0.01$; **$P < 0.001$.

Effects of TQS on IκB mRNA expression in the hippocampus

The effects of TQS on IκB mRNA expression in LPS-induced neuroinflammatory pain are shown in Figure 16. One way ANOVA showed that TQS significantly reduced

the expression of IκB mRNA ($F_{3,20}$ = 23.93; P < 0.0001). Multiple comparisons of means showed that LPS (1 mg/kg) significantly (P < 0.001) enhanced the expression of IκB mRNA indicating that LPS-induced neuroinflammatory pain is mediated through NF-κB activation. Furthermore, TQS (4 mg/kg) significantly (P < 0.001) reduced IκB mRNA expression in LPS-induced neuroinflammatory pain supporting the hypothesis that TQS decreased neuroinflammatory pain-like symptoms by decreasing NF-κB activation in the hippocampus.

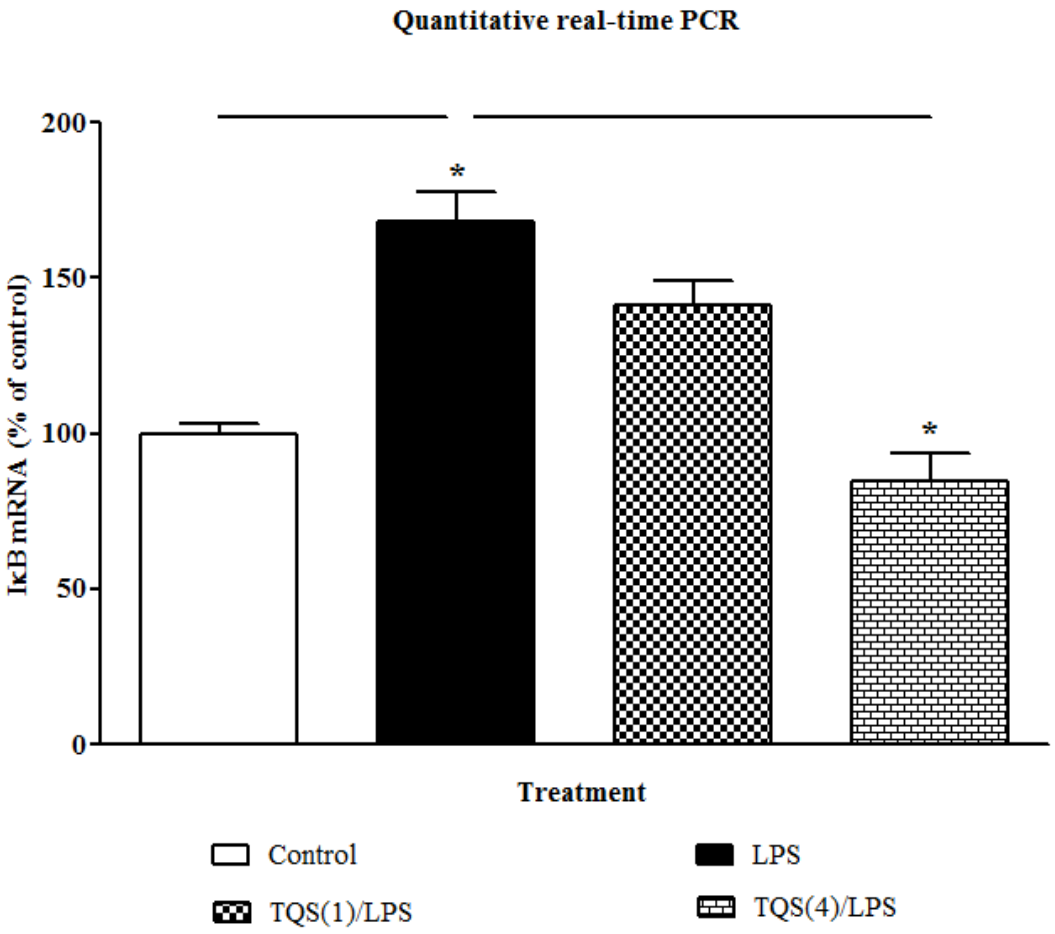

Figure 16: Lipopolysaccharide (LPS) significantly increased inhibitor of kappa B (IκB) mRNA expression compared to control. Moreover, TQS significantly reduced the expression of IκB mRNA compared to LPS treated group. Mice were given TQS (1 or 4 mg/kg, i.p.) 0.5 h before LPS administration. Control animals received equal volume of vehicle. Hippocampi were collected 6 h after LPS administration. The IκB mRNA expression was measured using quantitative real-time polymerase chain reaction. Data are expressed as mean $\pm$ S.E.M. (n = 6/group) $^{*}P < 0.001$.

Effects of co-administration of TQS and TEL on IκB mRNA expression in the hippocampus

The effects of co-administration of TQS and TEL on IκB mRNA expression are shown in Figure 17. Student's t-test showed that co-administration of TQS and TEL significantly ($P < 0.01$) reduced IκB mRNA expression which indicates that antiallodynic and antihyperalgesic effects of TQS are due to reduced activation of NF-κB.

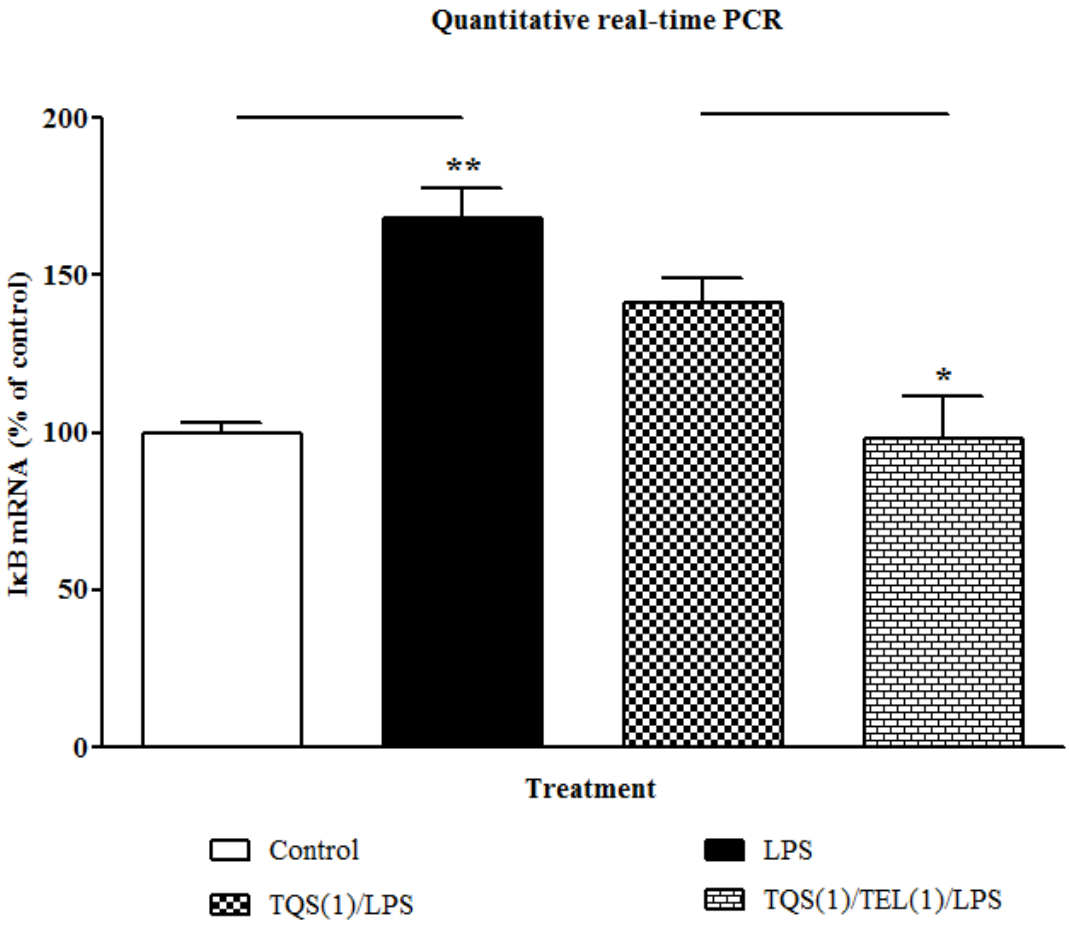

Figure 17: Lipopolysaccharide (LPS) significantly increased inhibitor of kappa B (IκB) mRNA expression compared to control. Moreover, telmisartan (TEL) in combination with TQS significantly reduced the expression of IκB mRNA compared to TQS treated group. Mice were given TQS (1 mg /kg, i.p.) 0.5 h before LPS administration. TEL (1 mg /kg, i.p.) was administered 5 min before TQS injection. Control animals received equal volume of vehicle. Hippocampi were collected 6 h after LPS administration. The IκB mRNA expression was measured using quantitative real-time polymerase chain reaction. Data are expressed as mean $\pm$ S.E.M. (n = 6/group). *P < 0.01; **P < 0.001.

Effects of MLA on TQS-induced decrease in IκB mRNA expression in the hippocampus

The effects of MLA on TQS-induced decrease in IκB mRNA expression in LPS-induced neuroinflammatory pain are shown in Figure 18. Student's t-test showed that MLA significantly ($P < 0.01$) reversed TQS-induced reduction in IκB mRNA expression. This validated that TQS-induced reduction in IκB mRNA expression is due to α7 nAChR.

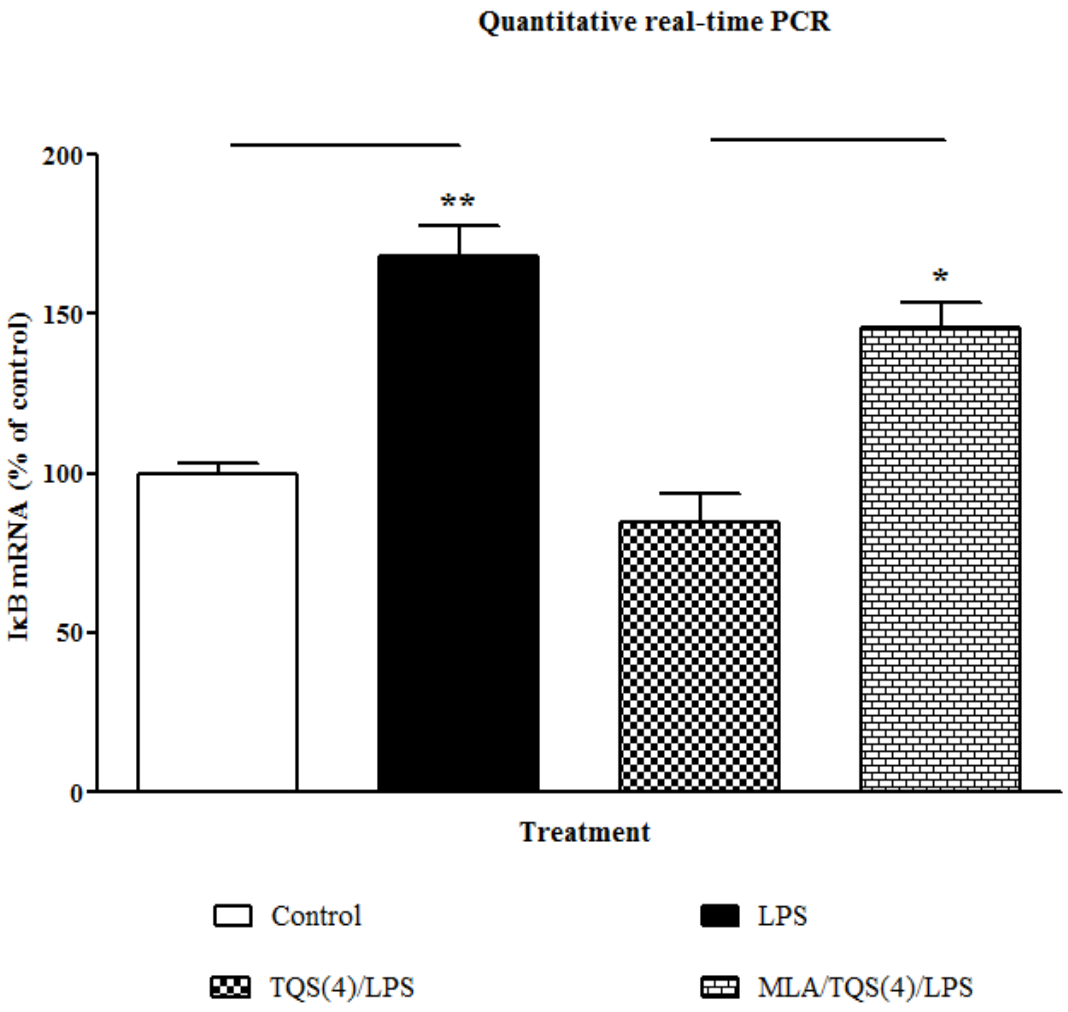

Figure 18: Lipolpolysaccharide (LPS) significantly increased inhibitor of kappa B (IκB) mRNA expression compared to control. Methyllcaconitine (MLA) significantly increased IκB mRNA expression compared to TQS treatment group. Mice were administered TQS (4 mg /kg, i.p.) 0.5 h before LPS administration. The MLA (3 mg /kg, i.p.) was given 10 min before TQS injection. Control animals received equal volume of vehicle. Hippocampi were collected 6 h after LPS administration. The IκB mRNA expression was measured using quantitative real-time polymerase chain reaction. Data are expressed as mean $\pm$ S.E.M. (n = 4-6/group). *$P < 0.01$; **$P < 0.0001$.

Effects of TQS on CD11b mRNA expression in the hippocampus

The effects of TQS on CD11b mRNA expression in LPS-induced neuroinflammatory pain are shown in the Figure 19. One way ANOVA showed that TQS significantly decreased CD11b mRNA expression in LPS-induced neuroinflammatory pain ($F_{3,14} = 10.36$; $P < 0.001$). Multiple comparisons of means showed that LPS significantly ($P < 0.01$) enhanced the expression of CD11b mRNA indicating the activation of microglia after LPS administration. Furthermore, TQS (4 mg/kg) significantly ($P < 0.01$) decreased CD11b mRNA expression indicative of reduced microglial activation in the hippocampus.

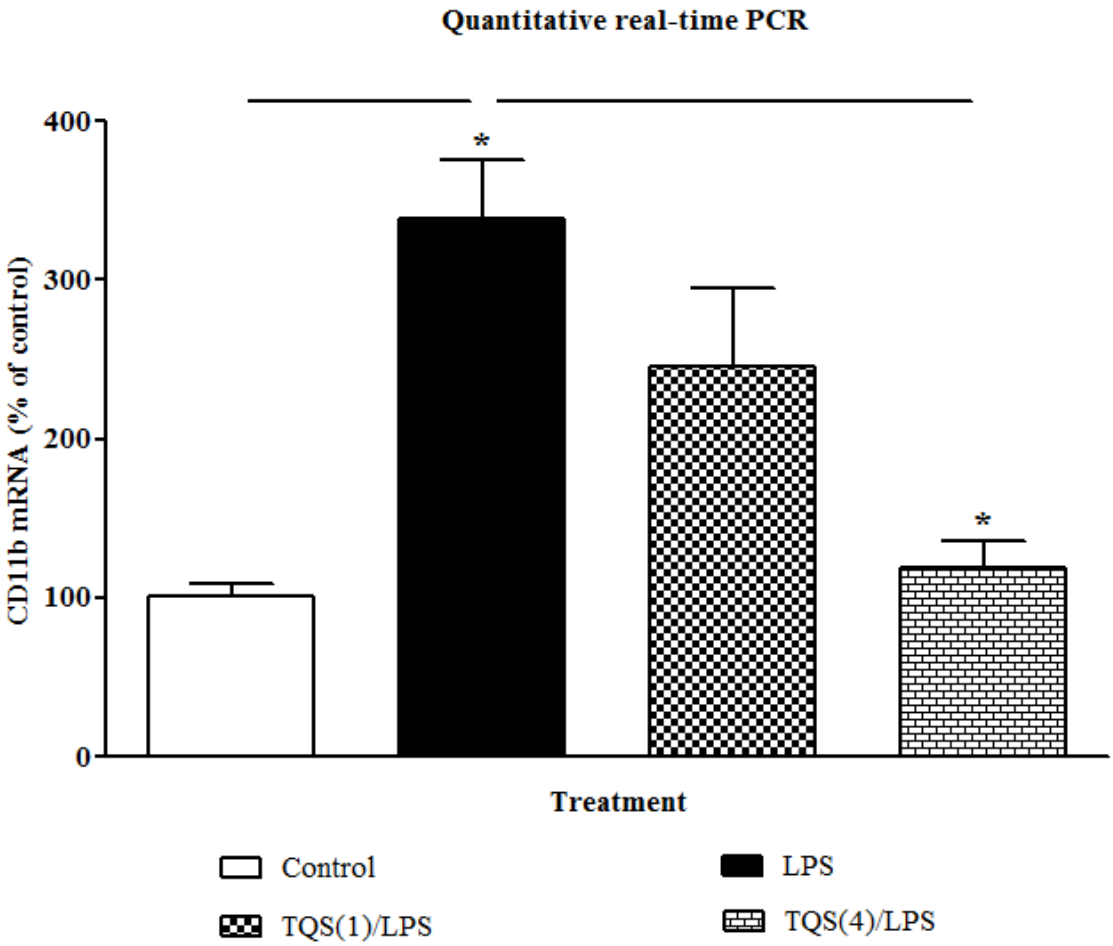

Figure 19: Lipopolysaccharide (LPS) significantly increased cluster of differentiation (CD)11b mRNA expression compared to control. Moreover, TQS significantly reduced the expression CD11b mRNA compared to LPS treated group. Mice were administered TQS (1 or 4 mg/kg, i.p.) 0.5 h before LPS administration. Control animals received equal volume of vehicle. Hippocampi were collected 6 h after LPS administration. The CD11b mRNA expression was determined using quantitative real-time polymerase chain reaction. Data are expressed as mean $\pm$ S.E.M. (n = 4-5/group) $^{*}P < 0.01$.

Effects of co-administration of TQS and TEL on CD11b mRNA expression in the hippocampus

The effects of co-administration of TQS and TEL on CD11b mRNA expression in LPS-induced neuroinflammatory pain are shown in the Figure 20. Student's t-test revealed that co-administration of TQS and TEL significantly ($P < 0.001$) decreased CD11b mRNA expression indicating reduced microglial activation in the hippocampus.

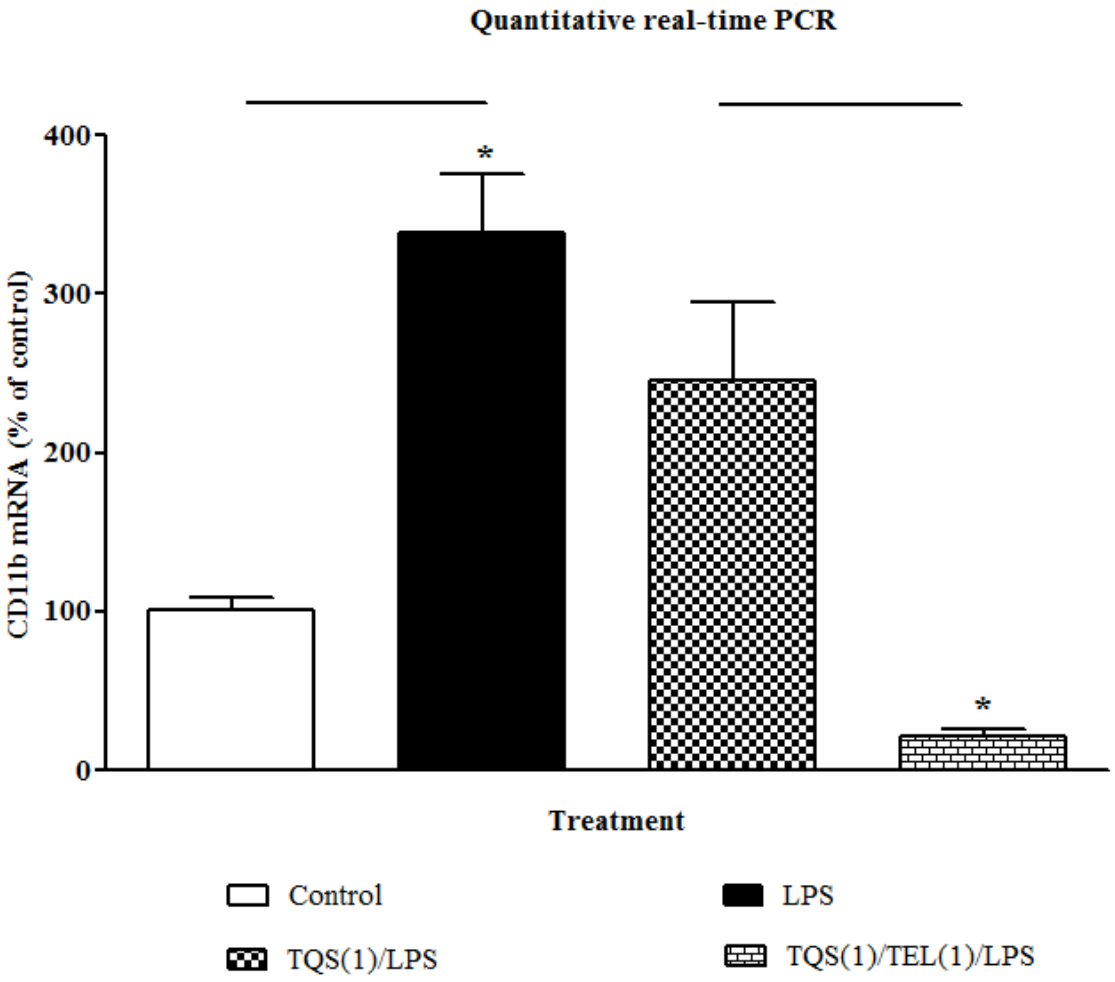

Figure 20: Lipopolysaccharide (LPS) significantly increased cluster of differentiation (CD)11b mRNA expression compared to control. Moreover, TQS in combination with telmisartan (TEL) significantly reduced the expression CD11b mRNA compared to TQS treated group. Mice were administered TQS (1 mg /kg, i.p.) 0.5 h before LPS administration. TEL (1 mg /kg, i.p.) was administered 5 min before TQS injection. Control animals received equal volume of vehicle. Hippocampi were collected 6 h after LPS administration. The CD11b mRNA expression was quantified using quantitative real-time polymerase chain reaction. Data are expressed as mean $\pm$ S.E.M. (n = 4-5/group). $^{*}P < 0.001$.

Effects of MLA on TQS-induced decrease in on CD11b mRNA expression in the hippocampus

The effects of MLA on TQS-induced decrease in CD11b mRNA expression in LPS-induced neuroinflammatory pain are shown in Figure 21. Student's t-test showed that MLA significantly ($P < 0.001$) reversed reduction in the expression of CD11b mRNA. This validated that TQS-induced reduction in CD11b mRNA is due to α7 nAChR.

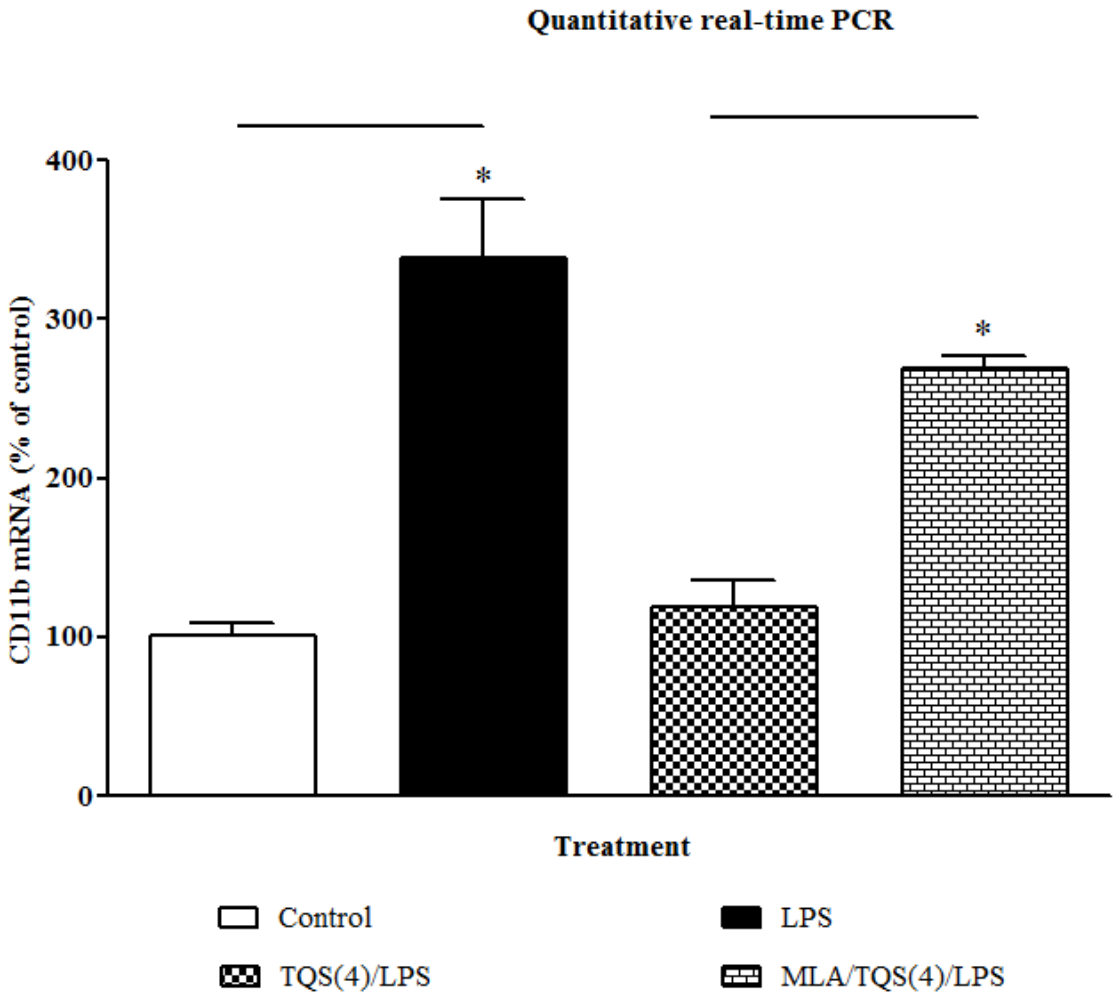

Figure 21: Lipolpolysaccharide (LPS) significantly increased cluster of differentiation (CD)11b mRNA expression compared to control. Methyllcaconitine (MLA) significantly increased CD11b mRNA expression compared to TQS treatment group. Mice were administered TQS (4 mg /kg, i.p.) 0.5 h before LPS administration. The MLA (3 mg /kg, i.p.) was given 10 min before TQS injection. Control animals received equal volume of vehicle. Hippocampi were collected 6 h after LPS administration. The CD11b mRNA expression was quantified using quantitative real-time polymerase chain reaction. Data are expressed as mean $\pm$ S.E.M. (n = 4-5/group). *$P < 0.001$.

Effects of TQS on the expression of p-NF-κB p^{65} in CA1 region of hippocampus

The effects of TQS on the expression of p-NF-κB p^{65} in CA1 are shown in Figure 22. One way ANOVA showed that TQS significantly decreased p-NF-κB p^{65} expression in LPS-induced neuroinflammatory pain ($F_{2,10} = 10.95$; $P < 0.01$). Multiple comparisons of means showed that LPS (1 mg/kg) significantly ($P < 0.05$) enhanced p-NF-κB p^{65} expression showing NF-κB activation after LPS administration. Furthermore, TQS (4 mg/kg) significantly ($P < 0.01$) decreased p-NF-κB p^{65} expression indicative of reduced NF-κB activation in CA1 region of hippocampus.

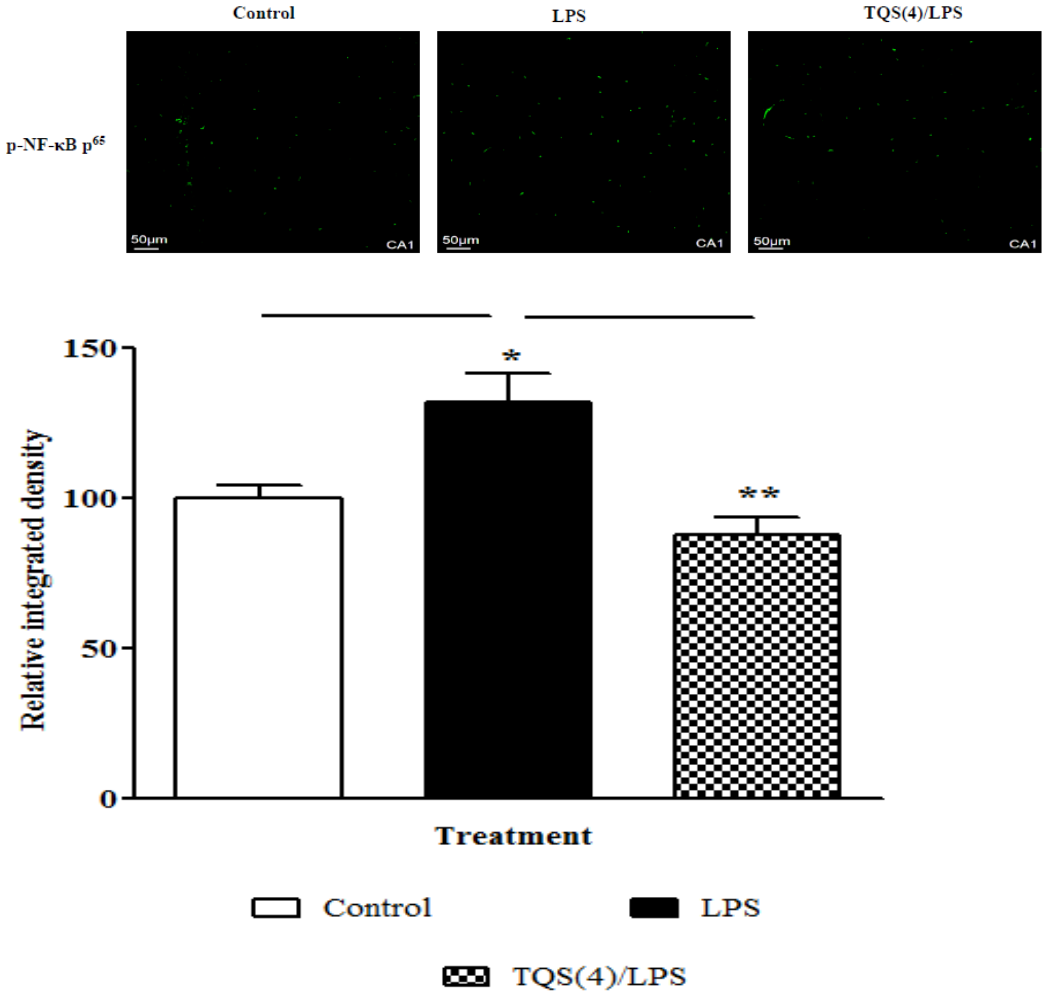

Figure 22: Lipopolysaccharide (LPS) significantly increased phospho-nuclear factor-κB (p-NF-κB) p^{65} expression compared to control in CA1 region of hippocampus. Moreover, TQS significantly reduced the expression of p-NF-κB p^{65} compared to LPS treated group. Mice were administered TQS (4 mg/kg, i.p.) 0.5 h before LPS administration. Control mice received equal volume of vehicle. Whole brain was collected 6 h after LPS administration. The p-NF-κB p^{65} expression was quantified using immunofluorescence assay. Data are expressed as mean $\pm$ S.E.M. Representative images show the immunofluorescence analysis in CA1 in hippocampus. Magnification 20 X, Scale bar = 50 um. (n = 4/group). $^{*}P < 0.05$; $^{**}P < 0.01$.

Effects of TQS on the expression of p-NF-κB p^{65} in DG in hippocampus

The effects of TQS on the expression of p-NF-κB p^{65} in DG region of the hippocampus are shown in Figure 23. One way ANOVA showed that TQS significantly decreased p-NF-κB p^{65} expression in LPS-induced neuroinflammatory pain ($F_{2,10}$ = 9.567; $P < 0.01$). Multiple comparisons of means showed that LPS (1 mg/kg) significantly enhanced ($P < 0.01$) p-NF-κB p^{65} expression indicating the activation of NF-κB after LPS administration. Furthermore, TQS (4 mg/kg) significantly ($P < 0.05$) decreased p-NF-κB p^{65} expression suggestive of reduced activation of NF-κB in DG region of hippocampus.

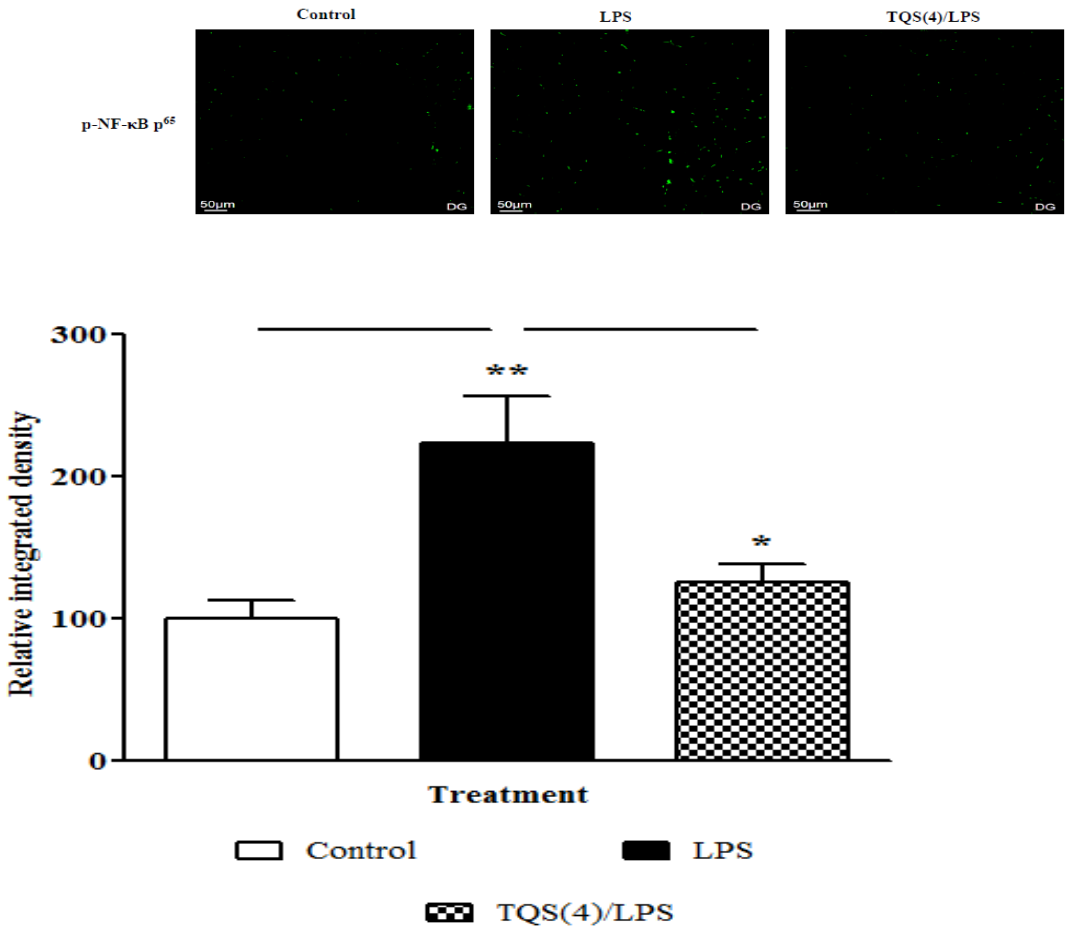

Figure 23: Lipopolysaccharide (LPS) significantly increased phospho-nuclear factor-κB (p-NF-κB) p^{65} expression compared to control in dentate gyrus (DG) region of hippocampus. Moreover, TQS significantly reduced the expression of p-NF-κB p^{65} compared to LPS treated group. Mice were administered TQS (4 mg/kg, i.p.) 0.5 h before LPS administration. Control mice received equal volume of vehicle. Whole brain was collected 6 h after LPS administration. The p-NF-κB p^{65} expression was quantified using immunofluorescence assay. Data are expressed as mean ± S.E.M. Representative images show the immunofluorescence in DG in hippocampus. Magnification 20 X, Scale bar = 50 um. (n = 5/group) $*P < 0.05$; $**P < 0.01$.

3.4. Discussion

The current study showed that pretreatment of TQS decreased IκB mRNA expression, a downstream marker the expression of which increases when NF-κB is activated and reduces when NF-κB becomes inactivated, in the hippocampus. This indicates that TQS causes NF-κB inactivation in LPS-induced neuroinflammatory pain that is likely linked with antiallodynic and antihyperalgesic effects in LPS-induced neuroinflammatory pain reported earlier (Abbas and Rahman, 2016). Moreover, co-administration of TEL, an NF-κB inhibitor, and TQS decreased the expression of IκB mRNA and LPS-induced tactile allodynia and thermal hyperalgesia associated with active NF-κB in LPS-induced neuroinflammatory pain. TQS also reduced immunoreactivity of p-NF-κB p^{65} both in DG and CA1 regions of the hippocampus. Furthermore, TQS decreased LPS-induced microglial activation due to reduced CD11b mRNA, a microglial activation marker, expression in the hippocampus. Pretreatment of MLA, an α7 nAChR antagonist, reversed TQS-induced reduction in the expression of IκB mRNA and CD11b mRNA in the hippocampus showing the involvement of α7 nAChR. Taken together, these results indicate that TQS decreases LPS-induced neuroinflammatory pain by decreasing hippocampal microglial activation due to reduced NF-κB activation involving microglial α7 nAChR (Figure 24).

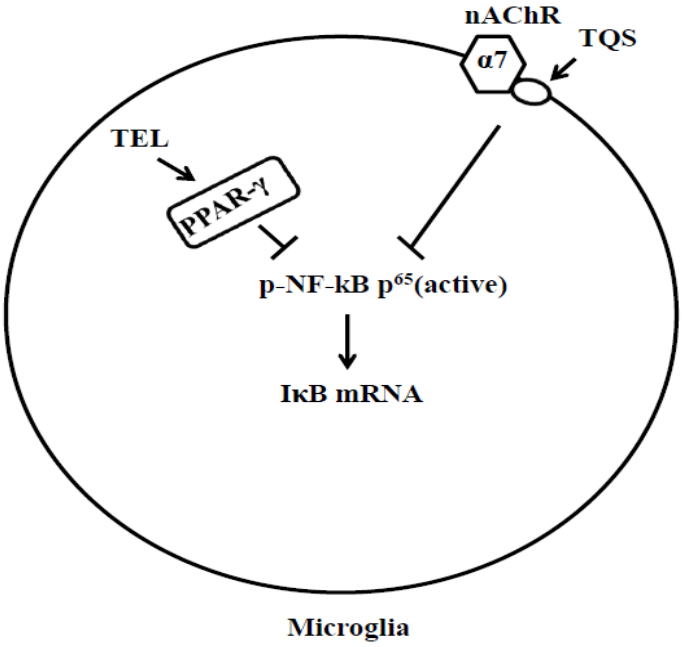

Figure 24: Proposed schematic of TQS effects against neuroinflammatory pain. TQS acts through microglial α7 nAChR positive allosteric modulatory site in the hippocampus. This results in reduced NF-κB activation through decreased microglial activation. Inhibition of NF-κB activity with TQS reduces the expression of IκB mRNA and neuroinflammatory pain-like symptoms.

The data presented in the current study are in line with the evidence that active NF-κB in microglial cells is a key mediator in LPS-induced neuroinflammatory pain model (Abbas and Rahman, 2016). The high level of IκB mRNA, a downstream mediator that indicates NF-κB activation, in the CNS is associated with development of neuroinflammatory pain-like symptoms (Loram et al., 2010). Moreover, the NF-κB activation linked with pain hypersensitivity has been characterized in other models of pain (Bethea et al., 1998; Ma and Bisby, 1998; Wilms et al., 2003) and is likely involved in increased pain sensitivity. Intrathecal administration of HIV-1 envelope glycoprotein

was shown to enhance NF-κB activation in the CNS in inflammatory model of pain (Milligan et al., 2000). Our findings of TQS effects on pain phenotypes and the down-regulation of the NF-κB activity provide credence to the above results and validate a link between active NF-κB and increased pain sensitivity in LPS-induced neuroinflammatory pain model in our mice model.

Our results showed that TQS in combination with TEL had synergistic effects on reduced I-κB mRNA expression and associated tactile allodynia and thermal hperalgesia indicating the utilization of NF-κB in activated microglial cells during antiallodynic and antihyperalgesic effects. It is important to note that TEL was found to reduce LPS-induced activation of NF-κB within microglial-like cells (Balaji and Ramanathan, 2014; Saavedra, 2012). In addition, TEL was shown to have efficacy in neuropathic pain (Jaggi and Singh, 2011) in animal model.

While TEL is regarded as an inhibitor of NF-kB, it is known to antagonize an angiotensin II receptor and is indicated in the treatment of hypertension. The drug also works as a partial agonist at peroxisome proliferator-activated receptor-gamma (PPAR-γ), a nuclear transcription factor (Haraguchi et al., 2010). Additionally, TEL exerts anti-inflammatory actions by reducing the expression of pro-inflammatory genes. These effects of TEL are mediated due to its PPAR-γ receptor activation property (Bernardo and Minghetti, 2006; Kapadia et al., 2008). Therefore, TEL-induced antiallodynic or antihyperalgesic effects are likely due to decreased activation of NF-κB in the hippocampus linked with PPAR-γ activation in hippocampal microglia. However, additional studies are required to validate the specific role of PPAR-γ associated with TEL actions in neuroinflammatory pain model.

The pretreatment of MLA, an α7 nAChR antagonist, reduced TQS-induced reduction in the level of I-κB mRNA in the hippocampus confirming the involvement of α7 nAChRs in the inactivation of NF-κB. To the best of our knowledge, this is the first finding that showed that occupancy of microglial α7 nAChR positive allosteric modulatory sites in the hippocampus causes reduction in the activation of NF-κB in neuroinflammatory pain. We, therefore, propose that microglial NF-κB activation should be inhibited for the blockade of LPS-induced neuroinflammatory pain.

Previous studies have indicated that neurons in CA1 region of the hippocampus mediate responses to painful stimuli (Khanna and Sinclair, 1989; Martuscello et al., 2012). Furthermore, other studies showed that the hippocampal DG and CA1 region play very important in neural processing of persistent pain (Soleimannejad et al., 2007). Our findings support the concept that both CA1 and DG are critically involved in the development and maintenance of painful symptoms. Our results indicated that LPS enhanced the expression of p-NF-κB p^{65} in DG and CA1 in microglial cells in pain hypersensitivity. The TQS caused a significant reduction of p-NF-κB p^{65} in these regions indicating that TQS-induced antiallodynic and antihyperalgesic effects are due to reduced NF-κB activation in DG and CA1. However, additional studies are needed involving direct microinjection of LPS and/or TQS in the hippocampus.

The fact that enhanced activation of NF-κB in the hippocampus is linked with increased microglial activation can be measured by increased CD11b mRNA expression in neuroinflammatory pain. Previously, we showed that TQS reduces the expression of Iba-1 in the hippocampus indicating the involvement of active microglia in LPS-induced neuroinflammatory pain (Abbas and Rahman, 2016). Previous studies showed that

neuroinflammatory pain is connected with neuropathic pain through microglial activation and other mediators (Ellis and Bennett, 2013; Jha et al., 2012; Zhuo et al., 2011). We propose that NF-κB positive cells are microglial cells that are highly involved in inflammatory process and facilitate painful behavior in animal models. It is possible that activated microglial cells in these regions of the hippocampus hypersensitize the neurons that mediate increased pain sensitivity. Therefore, antiallodynic and antihyperalgesic effects of TQS on α7 nAChR positive allosteric modulatory sites within the hippocampus are likely due to microglial cells, as LPS-induced enhanced CD11b mRNA expression was significantly reduced by TQS.

The co-administration of TQS and TEL resulted in decreased LPS-induced enhanced expression of CD11b mRNA. Pretreatment of MLA reversed TQS-induced reduction in the expression of CD11b mRNA suggesting activation of α7 nAChR decreases microglial activation in the hippocampus. Activation of NF-κB within the microglial cells enhances the expression of proinflammatory cytokines and pain mediators involving NF-κB-mediated gene expression that plays a critical role in painful behavior (Ferrini and De Koninck, 2013; Loram et al., 2010; Miwa et al., 1997). These findings suggest that reduction in micoglial and NF-κB activation in the hippocampus due to TQS is possibly associated with reduced tactile alloydynia and thermal hyperalgesia in this neuroinflammatory pain model. However, additional studies are warranted to determine the role of proinflammatory cytokines and other pain mediators, including BDNF in LPS-induced neuroinflammatory pain in the hippocampus.

The role for α7 expression and α7-targeting ligands in the cholinergic regulation of inflammation is highly established (de Jonge and Ulloa, 2007), the exact way in which

this connection is linked is not clearly known (Skok, 2009). Conventional thinking might presume that ligands such as GTS-21, an α7 partial agonist that has been shown in numerous research studies to be an effective regulator of neuroinflammation (Bruchfeld et al., 2010; Giebelen et al., 2007a, b; Hu et al., 2014; Kageyama-Yahara et al., 2008; Kox et al., 2009; Pavlov et al., 2007; Rosas-Ballina et al., 2009; Thomsen and Mikkelsen, 2012; van Westerloo et al., 2006; Wongtrakool et al., 2007; Wu et al., 2014; Yue et al., 2015), regulate neuroinflammation involving receptors' ion channel activity. However, even with the most effective agonists, under the best of conditions, α7 nAChR have a very low possibility of channel activation (Williams et al., 2012) and the critical screening of α7 agonists with calcium flux measurement requires that the drugs should be given with a type II PAM in order to overcome the α7 nAChR desensitization (Roncarati et al., 2008a). These findings would support the hypothesis that TQS in our experiments is working in the presence of endogenous ACh and/or choline to affect microglial function via channel-mediated calcium signals.

The nAChR in non-neuronal cells, including microglial cells might be processed differently from those in excitable cells such as neurons (Beckmann and Lips, 2013; Gahring and Rogers, 2005) and previous studies of α7 nAChR effects on microglial signal transduction (Hua et al., 2014; Morioka et al., 2015; Thomsen and Mikkelsen, 2012) fail to show any proof for α7-mediated ion currents. These observations lend credence to an alternative hypothesis that α7 nAChR might work as metabotropic receptors, modulating signal transduction pathways by the induction of specific non-conducting states associated with the receptor's cellular interactome (Kabbani et al.,

2013; King et al., 2015; Mulcahy et al., 2015; Paulo et al., 2009) which are due to unique features of α7's intracellular domain (Stokes et al., 2015).

The concept that the activity of α7 nAChR in reducing neuroinflammatory pain is independent of ion-channel activation is further validated by the finding that NS6740, which is primarily a channel desensitizer, is efficacious in numerous pain models (Papke et al., 2015) as the TQS analog GAT107 (Bagdas et al., 2016) which is a allosteric agonist and a very effective Type II PAM. It is, therefore, very likely that the effects of TQS in reducing neuroinflammatory pain are due to the direct induction of specific non-conducting α7 conformational states arising from the binding of the PAM at allosteric site and initiating downstream signaling pathway.

An interesting question would be to what extent the activity of ligands at the α7 orthosteric sites plays a role in reducing neuroinflammatory pain by α7 PAMs. Concentrations of ACh in the brain are constantly changing and much of the ACh release is through volume instead of synaptic transmission (Descarries et al., 1997) so that ACh could easily reach microglial cells to act in the presence of systemically administered PAMs. Furthermore, choline, a selective activator of α7 (Papke et al., 1996) is ubiquitous in the brain at a concentration of around 10 μM and may rise to much higher concentrations at inflammation or sites of trauma (Farooqui and Horrocks, 1994; Jope and Gu, 1991; Klein et al., 1997; Scremin and Jenden, 1991; Tartaglia et al., 2002). In conclusion, our results indicate that LPS-induced tactile allodynia and thermal hyperalgesia are mediated by microglial NF-κB activation involving α7 nAChR positive allosteric modulation.

Chapter 4: Effects of α7 nAChR PAM on microglial TNF expression and NE level in the hippocampus

4.1. Introduction

We have observed that TQS reduces painful behavior in animal models involving reduced NF-κB activation in hippocampus (see chapter 2). Moreover, it has been shown that increasing TNF in the hippocampus induces neuroinflammatory pain-like symptoms in animal models (Covey et al., 2000; Ignatowski et al., 1999). A previous study indicated that sustained high level of TNF exclusively in the hippocampus induces neuroinflammatory pain-like symptoms (Martuscello et al., 2012). Moreover, i.c.v. injection of recombinant TNF produces neuroinflammatory pain-like symptoms within CNS (Covey et al., 2002; Ignatowski et al., 1999; Oka et al., 1996). Thus, increasing TNF in the hippocampus causes increased pain sensitivity (Shubayev and Myers, 2000). We, therefore, propose that inhibition of TNF in the hippocampus might elicit reduced painful symptoms due to increased NE level involving α7 nAChRs and α2 adrenergic receptors.

Previously, it was shown that hippocampus is innervated extensively with pre-synaptic α2-adrenergic receptors that play very important role in the release of NE involving TNF (Kiss et al., 1995; Scheinin et al., 1994). For example, in neuroinflammatory pain-like condition, the release of NE in the hippocampus is reduced due to elevated level of TNF (Covey et al., 2000; Ignatowski et al., 1999). Taken together, the above observation suggests that reduction in the release of NE associated with high level of TNF in the hippocampus results in neuroinflammatory pain-like symptoms. We, therefore, propose that reducing TNF and increasing NE level within

hippocampus through microglial α7 nAChRs positive allosteric modulation might be potential therapeutic strategy for neuroinflammatory pain.

Idazoxan (IDA) (Remko et al., 2006), a selective α2 receptor antagonist, has high affinity at α2 adrenergic receptor (Walter et al., 1984). The IDA is a potent α2 adrenergic receptor blocker (Fong et al., 2000). Due to its enhanced lipid solubility (Fong et al., 2000; Remko et al., 2006), the IDA is expected to cross BBB. The IDA is used to characterize the involvement of α2 adrenergic receptor.

Previously we have shown that TQS reduced LPS-induces neuroinflammatory pain due to decreased microglial and NF-κB activation in the hippocampus involving α7 nAChRs. However, the effects of TQS on TNF expression and NE release in the hippocampus are not investigated in LPS-induced neuroinflammatory pain. Given the previous reports, it is probable that reducing TNF and NE in the hippocampus could be of therapeutic utility for reducing neuroinflammatory pain-like symptoms involving α7 nAChR. Therefore, we hypothesized that TQS will reduce painful behavior by reducing TNF and increasing NE level in the hippocampus. In the present study, we have determined the effects of TQS on TNF expression and NE release in the hippocampus in mice. We also determined the effects of TQS on microglial morphology in the hippocampus.

4.2. Methods

Animals

Male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed under standard laboratory conditions as mentioned before (see chapter 2, section 2.2).

Drug treatment

The IDA was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in normal saline (0.9% NaCl). The IDA (2 mg/kg, i.p.) was administered 1 h prior to measurement of tactile allodynia and thermal hyperalgesia. The source, method of reconstitution, and time of administration for other drugs used in this study are described earlier (see chapter 2, section 2.2). Control animals were administered an equal volume of vehicle. The dose for IDA was selected based previous report (Andurkar et al., 2012).

Tactile allodynia

Tactile allodynia was measured as described previously (see chapter 2, section 2.2).

Thermal hyperalgesia

Thermal hyperalgesia was quantified as described before (see chapter 2, section 2.2).

RNA isolation and cDNA synthesis

Total RNA was isolated from hippocampal tissue as described previously (see chapter 3, section 3.2).

Quantitative real-time PCR

Quantitative real-time PCR was performed as described earlier (see chapter 3, section 3.2). Primers sequences were obtained from Integrated DNA Technologies (Coralville, Iowa, USA) and are displayed in Table 2.

Table 2: Prime primer sequence for GAPDH, and TNF

Gene	**Primer sequence (5'-3')**
GAPDH	GTGGAGTCATACTGGAACATGTAG (forward)
	AATGGTGAAGGTCGGTGTG (reverse)
TNF	TCTTTGAGATCCATGCCGTTG (forward)
	AGACCCTCACACTCAGATCA (reverse)

Enzyme-linked immunosorbent assay (ELISA)

Mice were sacrificed six h after LPS administration through rapid decapitation and their hippocampi were dissected out. Hippocampal tissues were stored at -80^{o}C until further analysis. Brain tissue samples were then homogenized in tris buffer saline (1X) containing protease inhibitor mix (complete, Mini, Roche, Indianapolis, IN, USA) and 1% triton X-100. The hippocampal tissue homogenates were then centrifuged at 17000×g at 4^{o}C for 30 min. The supernatants were collected and stored at −80°C until further analysis. The hippocampal TNF was measured by ELISA kit (eBioscience), according to manufacturer's instructions.

NE assay

The NE assay was carried out as described earlier with minor modification (Roni and Rahman, 2013). Briefly, mice were sacrificed by rapid decapitation six h after LPS administration. Their hippocampi were dissected from 1-mm coronal sections using

Allen Brain Atlas and mouse brain stereotaxic coordinates. The brain tissue samples were stored at -80°C until further analysis. For NE assay, hippocampi were diluted with 0.1N perchloric acid to 5 volumes of their weight (1:5 as g/ml); samples were then homogenized and centrifuged at 14,000 ×g for 30 min at 4 °C. Twenty µl of resulting supernatant was injected onto a high performance liquid chromatography (HPLC) system coupled with electrochemical detection unit (ESA Inc., Chelmsford, MA, USA) to determine NE. The HPLC system comprised of a solvent delivery module, a Coulochem III detector equipped with a 5011A analytical cell and 5020 guard cell. The potential for guard cell was set at +350 mV. The gain of the detector was set at 100 nA for both electrodes (electrode 1: −150 mV, electrode 2: +220 mV). The mobile phase contained 75 mM NaH_2PO_4, 1.7 mM 1-octane sulfonic acid, 25 µM EDTA, 100 µL/L triethylamine, 100 mL/L of acetonitrile. The pH of mobile phase was adjusted to 3.0 using O-phosphoric acid. The C-18 analytical column (BetaBasic-18 column, 150×3 mm, Thermo Hypersil-Keystone, PA, USA) was used as stationary phase and the flow rate was maintained at 0.15 mL/min during NE assay. Data were collected in ESA chromatography data system (EZ Chrom Elite, Chelmsford, MA, USA). Peak's area under the curve and calibration factors based on the standard solutions (1–100 ng/mL) were used to determine the quantity of NE and the values are expressed as ng/mg weight of hippocampal tissue.

Immunofluorescence assay

Immunofluorescence assay was performed as described before (see chapter 3, section 3.2) using primary antibody for TNF (Santa Cruz Biotech, Dallas, Texas, USA)

and secondary antibody labeled with FITC (1:50) (Santa Cruz Biotechnology, Dallas, TX, USA).

Immunohistochemistry

Immunohistochemistry was performed as described earlier with some modification (Silverman et al., 2014). Briefly, mice were sacrificed six after LPS administration through rapid decapitation. Their brains were then harvested and fixed with 4% paraformaldehyde (Acros organics, New Jersey, USA) for 24 h. Mice brains were then cryoprotected at 4^{o}C in 30% sucrose (Sigma-Aldrich, St. Louis, MO, USA) for 24 h/until brain sank. The 40 μm coronal sections were cut using Leica cryostat. Free floating sections were then placed in water bath maintained at 90^{o}C having boiling 0.01 M citrate buffer (pH 6.0) for 10 min, followed by quenching in 0.3% hydrogen peroxide (Acros organics, New Jersey, USA) in methanol for 5-10 min. Hippocampal tissue sections were then blocked in 1.5% normal goat serum (Santa Cruz Biotech, Dallas, Texas, USA) at room temperature for 1 h. Free floating sections were then incubated with primary antibody (goat anti-raabit-Iba-1; 2.25:1000, Wako, Osaka, Japan) at room temperature for 1.5 h. Sections were then incubated in secondary antibody at room temperature for 30 min (biotinylated goat anti-rabbit; 5:1000, Santa Cruz Biotech, Dallas, Texas, USA), followed by staining with avidin-biotin complex, diaminobenzidine, and mounted on superfrost plus microscope slides (Fisher Scientific, USA). Images were taken at 20X after overnight drying with bright field microscopy. A negative control omitting the primary antibody was also carried out. Three to four slices were processed per each animal used. To quantify Iba-1-positive microglial cells across different treatments, three to four random fields in the hippocampus were taken.

Quantification of % area occupied by microglial cells was carried out using Image J software.

Statistical analyses

For NE assay, ELISA, immunofluorescence assay, and quantitative real-time PCR, data were analyzed by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. For quantitative real-time PCR for MLA and TQS/IDA combination data for thermal hyperalgesia, Student's t-test was used. For tactile allodynia, Mann-Whitney test for TQS/IDA combination was applied using GraphPad Prism 5.0 (GraphPad Inc., San Diego, CA, USA). The difference between treatments was considered as significant at $P < 0.05$. All results are expressed as mean ± S.E.M.

4.3. Results

Effects of TQS on TNF mRNA expression in the hippocampus

The effects of TQS on TNF mRNA expression are displayed in Figure 25**.** One way ANOVA showed that TQS significantly reduced TNF mRNA expression ($F_{3,12} = 23.78$; $P < 0.0001$). Multiple comparisons of means revealed that LPS (1 mg/kg) significantly ($P < 0.001$) enhanced TNF mRNA expression suggesting LPS-induced neuroinflammatory pain is due to enhanced TNF mRNA expression. Furthermore, TQS (4 mg/kg) significantly ($P < 0.01$) reduced the expression of TNF mRNA in LPS-induced neuroinflammatory pain. This supports our hypothesis that TQS decreases neuroinflammatory pain-like symptoms by reducing the expression of TNF mRNA in the hippocampus.

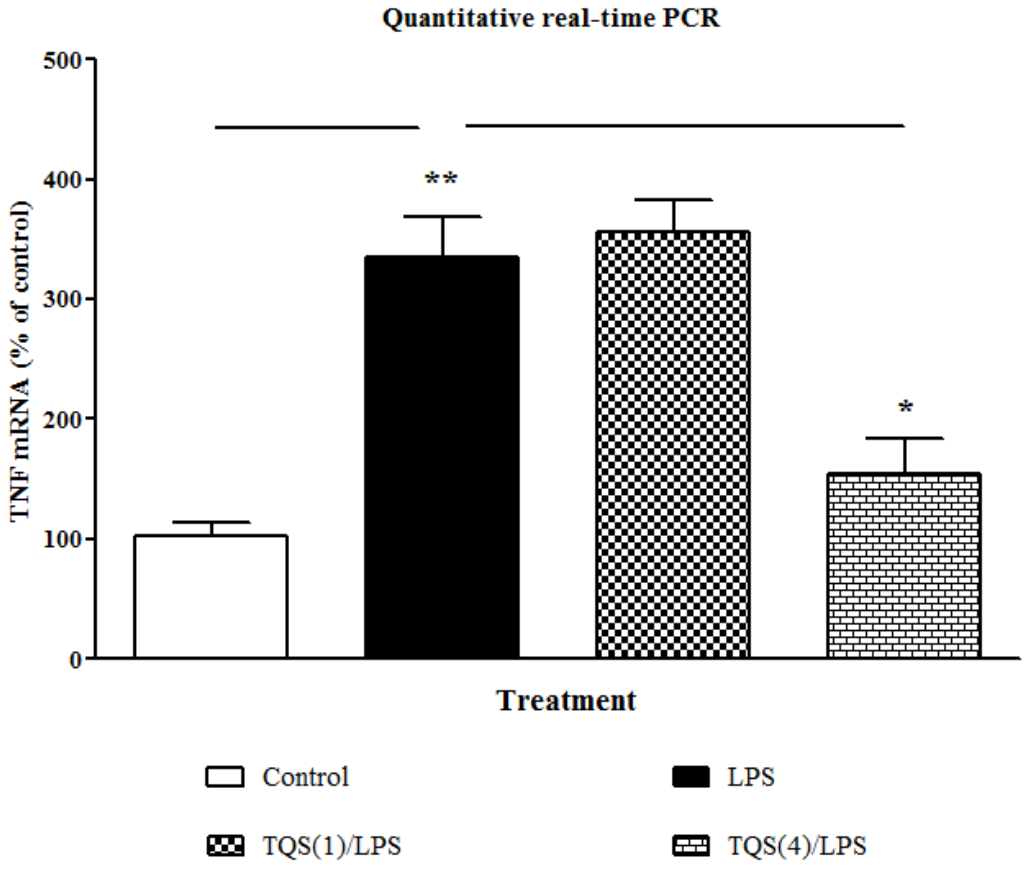

Figure 25: Lipopolysaccharide (LPS) significantly increased tumor necrosis factor (TNF) mRNA expression compared to control. Moreover, TQS significantly reduced the expression of TNF mRNA compared to LPS treated group. Animals were administered TQS (1 or 4 mg/kg, i.p.) 0.5 h before LPS administration. Control animals received equal volume of vehicle. Hippocampi were collected 6 h after LPS administration. The TNF mRNA expression was quantified using quantitative real-time polymerase chain reaction. Data are expressed as mean $\pm$ S.E.M. (n = 4/group) *$P < 0.01$; **$P < 0.001$.

Effects of MLA on TQS-induced decrease in TNF mRNA expression in the hippocampus

The effects of MLA on TQS-induced reduced TNF mRNA expression are displayed in Figure 26. Student's t-test revealed that MLA significantly ($P < 0.01$) reversed the TQS-

induced reduction in the expression of TNF mRNA. This validated our hypothesis that TQS-mediated reduction in TNF mRNA expression is due to α7 nAChR.

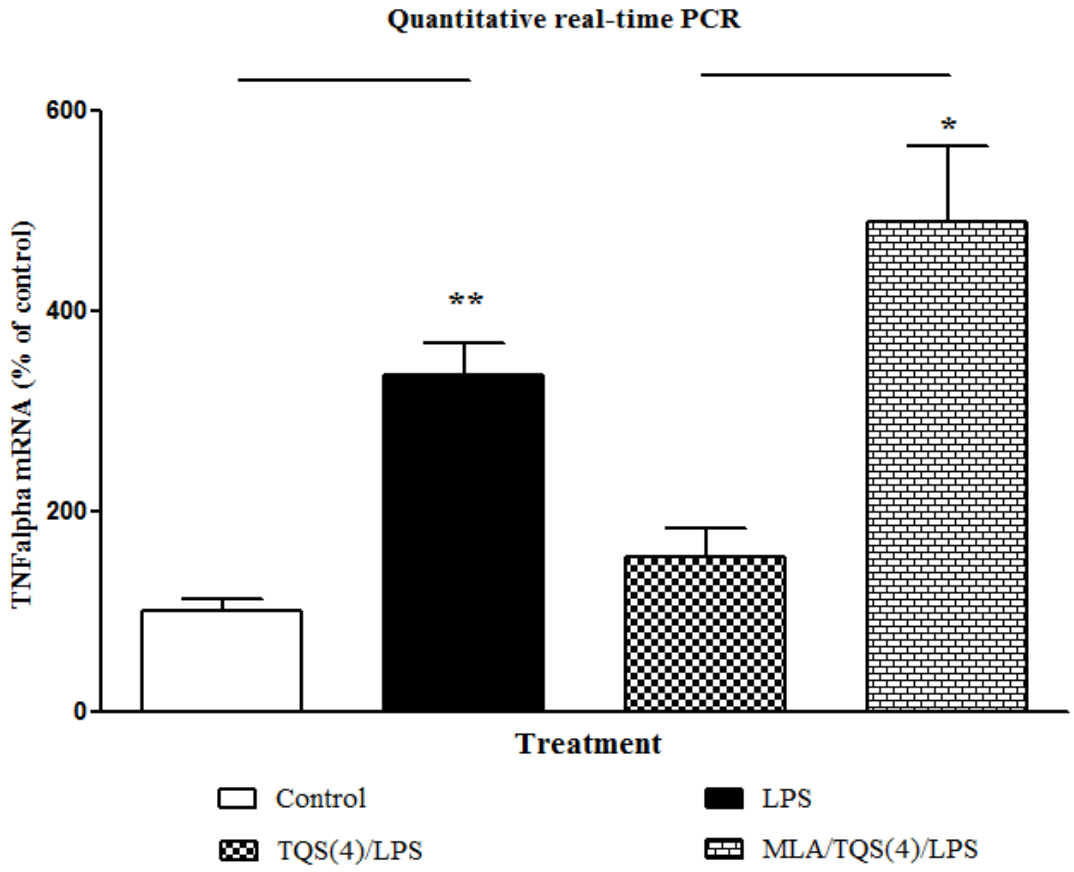

Figure 26: Lipolpolysaccharide (LPS) significantly increased tumor necrosis factor (TNF) mRNA expression compared to control. Methyllycaconitine (MLA) significantly increased TNF mRNA expression compared to TQS treatment group. Mice were administered TQS (4 mg /kg, i.p.) 0.5 h before LPS administration. The MLA (3 mg /kg, i.p.) was given 10 min before TQS injection. Control animals received equal volume of vehicle. Hippocampi were collected 6 h after LPS administration**.** The TNF mRNA expression was quantified using quantitative real-time polymerase chain reaction. Data are expressed as mean $\pm$ S.E.M. (n = 4/group). *$P < 0.01$; **$P < 0.001$.

Effects of TQS on TNF level in the hippocampus

The effects of TQS on TNF expression are displayed in Figure 27. One way ANOVA revealed that TQS significantly decreased TNF expression ($F_{3,21} = 43.45$; $P < 0.0001$). Multiple comparisons of means indicated that LPS (1 mg/kg) significantly ($P < 0.001$) enhanced TNF expression indicating LPS-induced neuroinflammatory pain is due to elevated expression of TNF. Moreover, TQS (1 or 4 mg/kg) significantly ($P < 0.001$) reduced the expression of TNF in LPS-induced neuroinflammatory pain indicating TQS decreased neuroinflammatory pain-like symptoms by reducing TNF expression in the hippocampus.

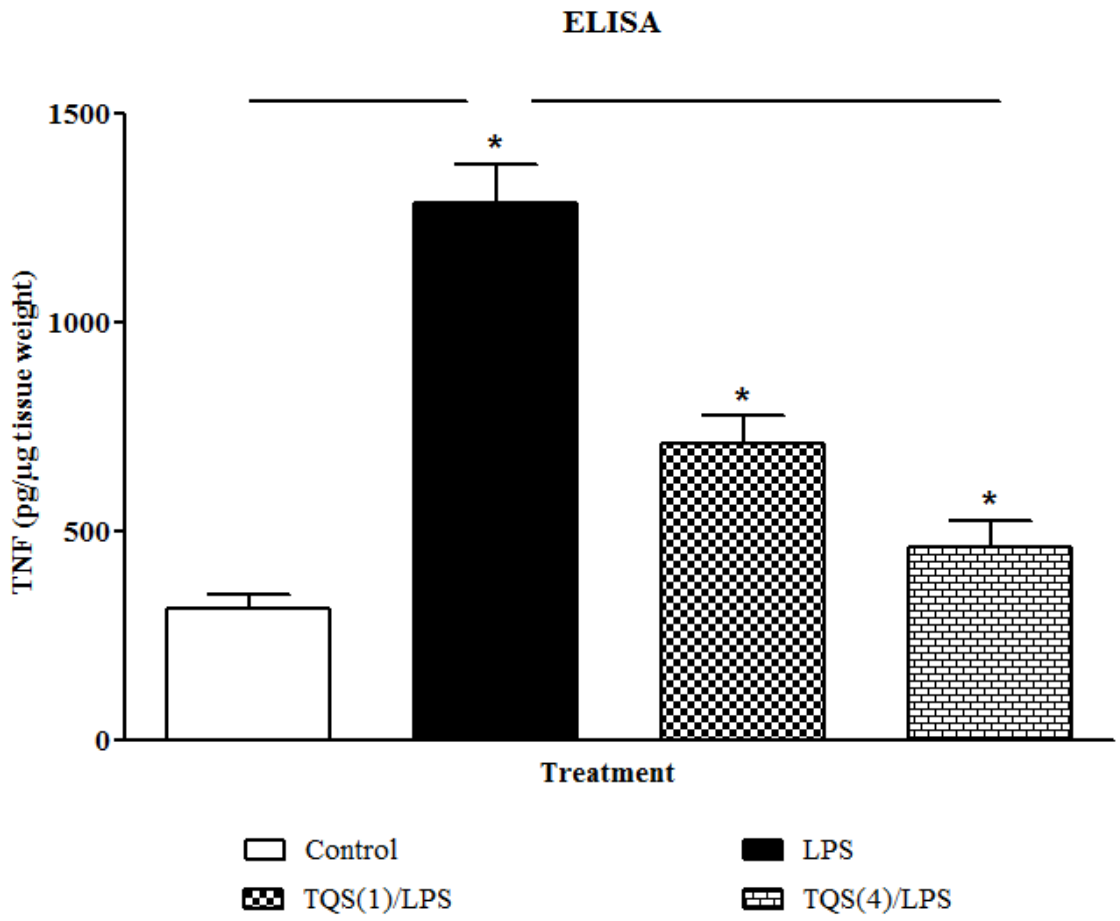

Figure 27: Lipopolysaccharide (LPS) significantly increased the expression of tumor necrosis factor (TNF) compared to control. Moreover, TQS significantly reduced the expression TNF compared to LPS treated group. Animals received TQS (1 or 4 mg/kg, i.p.) 0.5 h before LPS administration. Control animals received equal volume of vehicle. Hippocampi were collected 6 h after LPS administration. The TNF level was quantified using enzyme-linked immunosorbent assay. Data are expressed as mean ± S.E.M. (n = 6-7/group). *$P < 0.001$.

Effects of TQS on TNF expression in CA1 region of hippocampus

The effects of TQS on TNF expression in CA1 region of hippocampus are displayed in Figure 28. One way ANOVA showed that TQS significantly decreased the expression of TNF in LPS-induced neuroinflammatory pain ($F_{2,15} = 22.52$; $P < 0.0001$). Multiple comparisons of means showed that LPS (1 mg/kg) significantly ($P < 0.001$) enhanced the expression of TNF. Furthermore, TQS (4 mg/kg) significantly ($P < 0.001$) decreased the expression TNF in CA1 indicative of reduced TNF level in CA1 region of hippocampus during antiallodynic and antihyperalgesic effects of TQS.

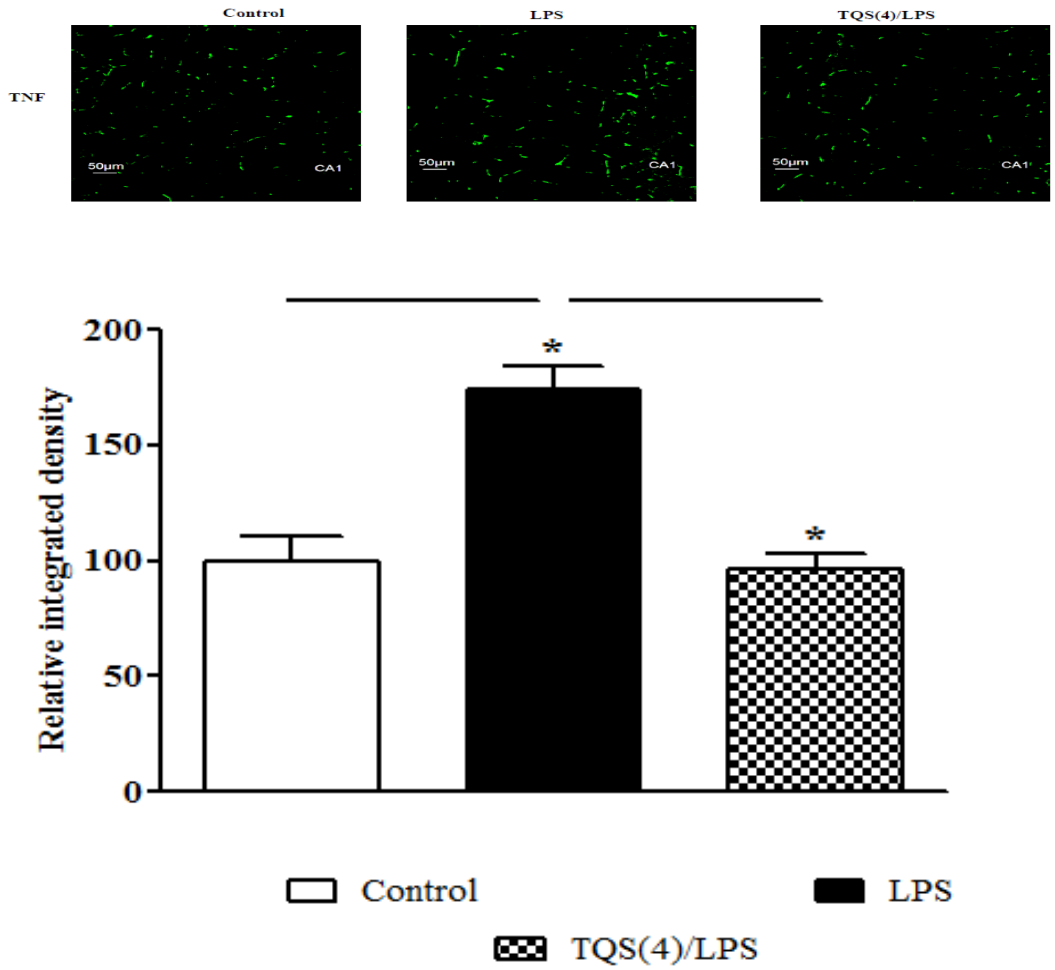

Figure 28: Lipopolysaccharide (LPS) significantly increased tumor necrosis factor (TNF) expression compared to control in CA1 region of hippocampus. Moreover, TQS significantly reduced the expression of TNF compared to LPS treated group. Mice were administered TQS (4 mg/kg, i.p.) 0.5 h before LPS administration. Control mice received equal volume of vehicle. Whole brain was collected 6 h after LPS administration. The TNF expression was quantified using immunofluorescence assay. Data are expressed as mean ± S.E.M. Representative images show the immunofluorescence in CA1 region in hippocampus. Magnification 20 X, Scale bar = 50 um. (n = 6/group). *$P < 0.001$.

Effects of TQS on TNF expression in DG in hippocampus

The effects of TQS on TNF expression in DG region of hippocampus are displayed in Figure 29. One way ANOVA indicated that TQS significantly reduced the expression of TNF in LPS-induced neuroinflammatory pain ($F_{2,15} = 28.55$; $P < 0.0001$). Multiple comparisons of means showed that LPS (1 mg/kg) significantly ($P < 0.001$) increased the expression of TNF. Furthermore, TQS (4 mg/kg) significantly ($P < 0.001$) reduced TNF expression suggestive of decreased TNF level in DG region of hippocampus during antiallodynic and antihyperalgesic effects of TQS.

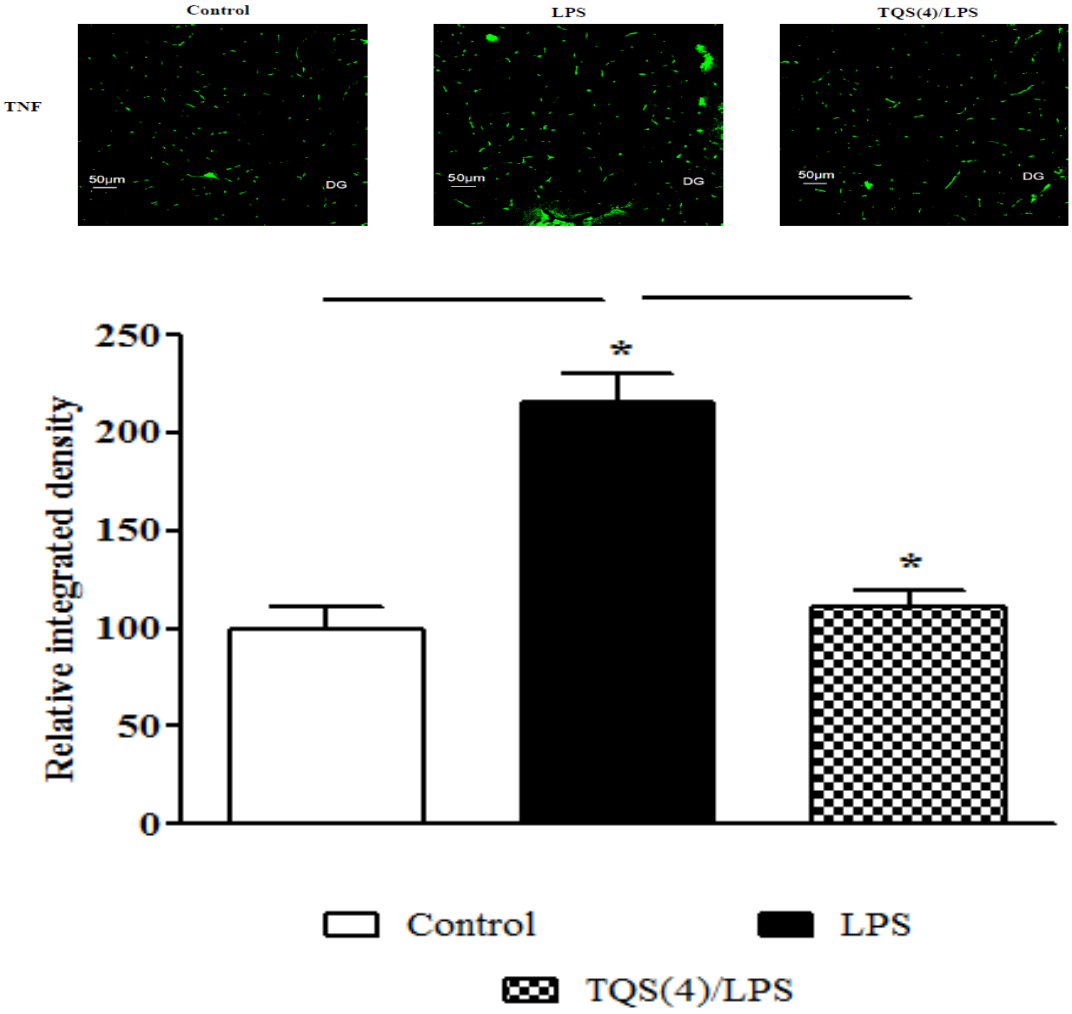

Figure 29: Lipopolysaccharide (LPS) significantly increased tumor necrosis factor (TNF) expression compared to control in dentate grus (DG) region of hippocampus. Moreover, TQS significantly reduced the expression of TNF compared to LPS treated group. Mice were administered TQS (4 mg/kg, i.p.) 0.5 h before LPS administration. Control mice received equal volume of vehicle. Whole brain was collected 6 h after LPS administration. The TNF expression was quantified using immunofluorescence assay. Data are expressed as mean $\pm$ S.E.M. Representative images show the immunofluorescence in DG in hippocampus. Magnification 20 X, Scale bar = 50 um. (n = 5-7/group). *$P < 0.001$.

Effects of IDA on TQS-induced antiallodynic response in von Fey filament test

The effects of IDA on TQS-induced antiallodynia are displayed in Figure 30. Mann-Whitney test showed that IDA significantly ($P < 0.05$) decreased 50% paw withdrawal threshold compared with TQS treated group indicating that reduction in the level of NE after IDA pretreatment.

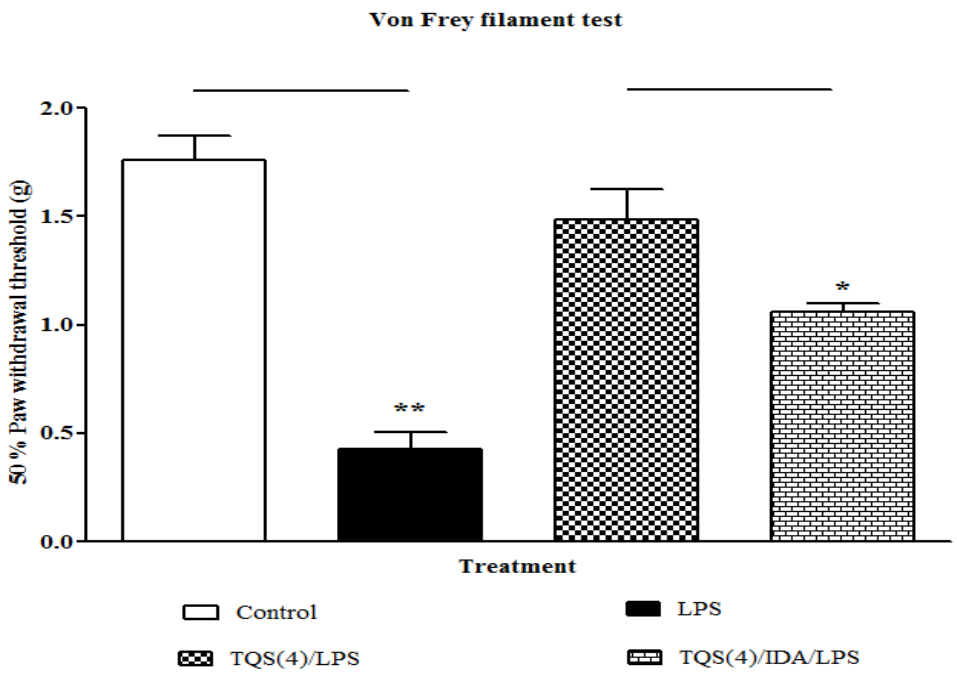

Figure 30: Lipopolysaccharide (LPS) significantly reduced 50% paw withdrawal threshold (tactile allodynia) compared to control. Moreover, idazoxan (IDA) significantly decreased 50% paw withdrawal threshold compared to TQS treated group. Mice received TQS (4 mg/kg, i.p.) 0.5 h before LPS administration. The IDA (2 mg/kg, i.p.) was administered 1 h before assessment of tactile allodynia. Control animals received equal volume of vehicle. Latency on hot plate was measured six h after LPS administration. Data are expressed as mean ± S.E.M. (n = 7-8/group). *$P < 0.05$; **$P < 0.01$.

Effects of IDA on TQS-induced antihyperalgesia in hot plate test

The effects of IDA on TQS-induced antihyperalgesia are displayed in Figure 31. Student's t-test showed that IDA significantly ($P < 0.05$) reduced hot plate latency time compared to TQS treated group indicating that antihyperalgesic effects of TQS are due to increased NE release in the hippocampus.

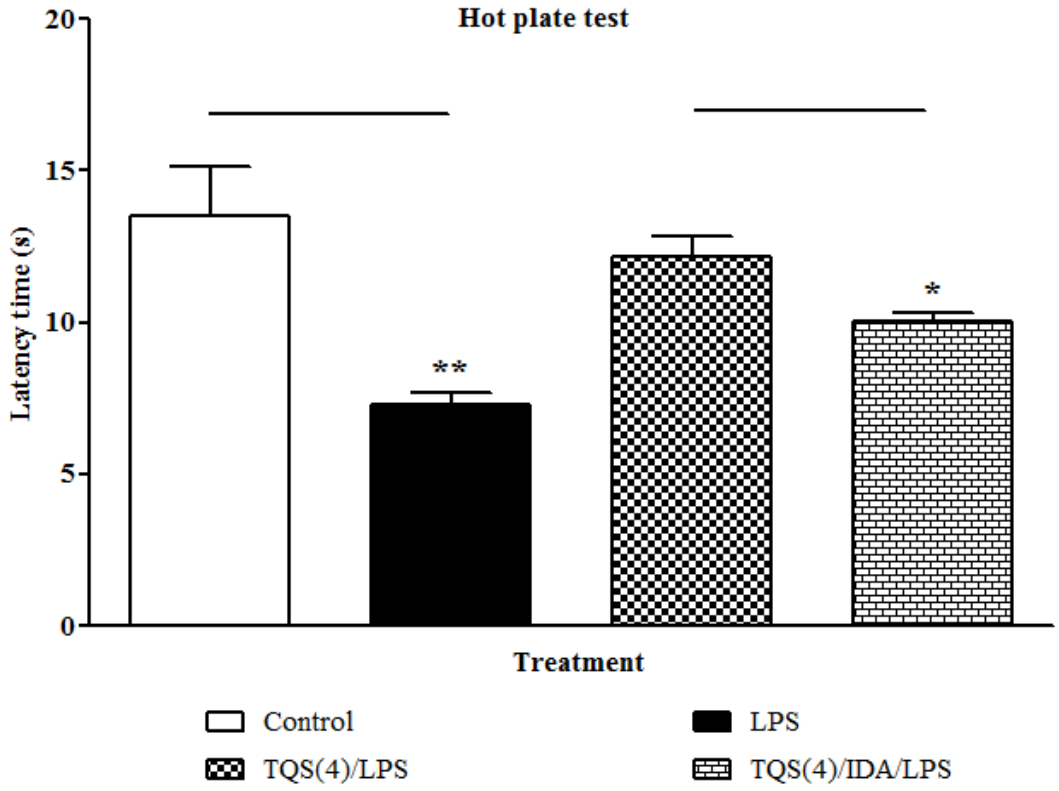

Figure 31: Lipopolysaccharide (LPS) significantly reduced latency time (thermal hyperalgesia) compared to control. Moreover, idazoxan (IDA) significantly decreased latency time compared to TQS treated group. Mice received TQS (4 mg/kg, i.p.) 0.5 h before LPS administration. IDA (2 mg/kg, i.p.) was administered 1 h before assessment of hyperalgesia. Control animals received equal volume of vehicle. Latency on hot plate was measured six h after LPS administration. Data are expressed as mean $\pm$ S.E.M. (n = 6/group). *P <0.05; **P < 0.01.

Effects of TQS on NE release in the hippocampus

The effects of TQS on NE release are displayed in Figure 32. One way ANOVA showed that TQS significantly changed NE level in the hippocampus ($F_{3,20} = 5.943$; $P <$ 0.01). Multiple comparisons of means showed that LPS (1 mg/kg) significantly ($P <$ 0.05) reduced the level of NE in the hippocampus indicating LPS-induced

neuroinflammatory pain is due to reduced NE level in the hippocampus. Furthermore, TQS (4 mg/kg) significantly ($P < 0.01$) elevated level of NE in LPS-induced neuroinflammatory pain suggesting that TQS decreased neuroinflammatory pain-like symptoms by increasing the level of NE in the hippocampus.

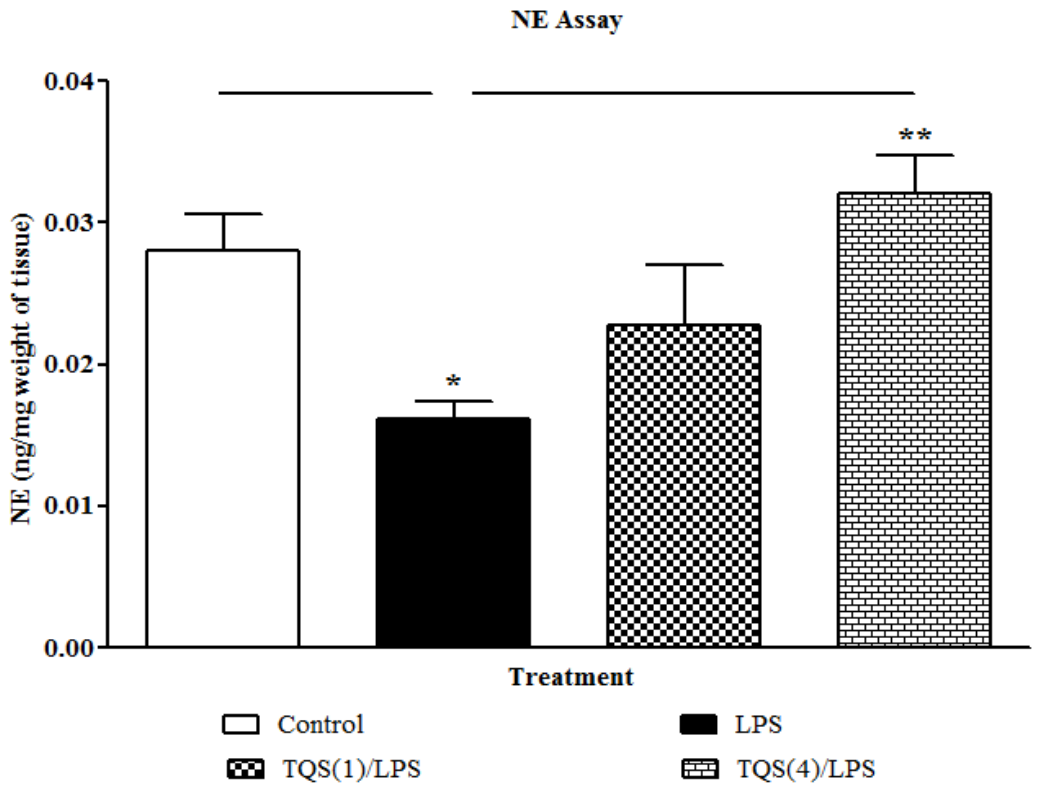

Figure 32: Lipopolysaccharide (LPS) significantly reduced norepinephrine (NE) level compared to control. Moreover, TQS significantly increased the level of NE compared to LPS treated group. Animals received TQS (1 or 4 mg/kg, i.p.) 0.5 h before LPS administration. Control animals received equal volume of vehicle. Hippocampi were collected 6 h after LPS administration. The NE level was measured by HPLC-EC. Data are expressed as mean $\pm$ S.E.M. (n = 5-7/group). *$P < 0.05$; **$P < 0.01$.

Effects of IDA on TQS-induced increase NE level in the hippocampus

The effects of IDA on TQS-induced increased level of NE are displayed in Figure 33. Student's t-test showed that IDA significantly ($P < 0.05$) reduced the elevated level of NE in the hippocampus. This validated that TQS-mediated elevated level of NE is due to α2 adrenergic receptor involvement.

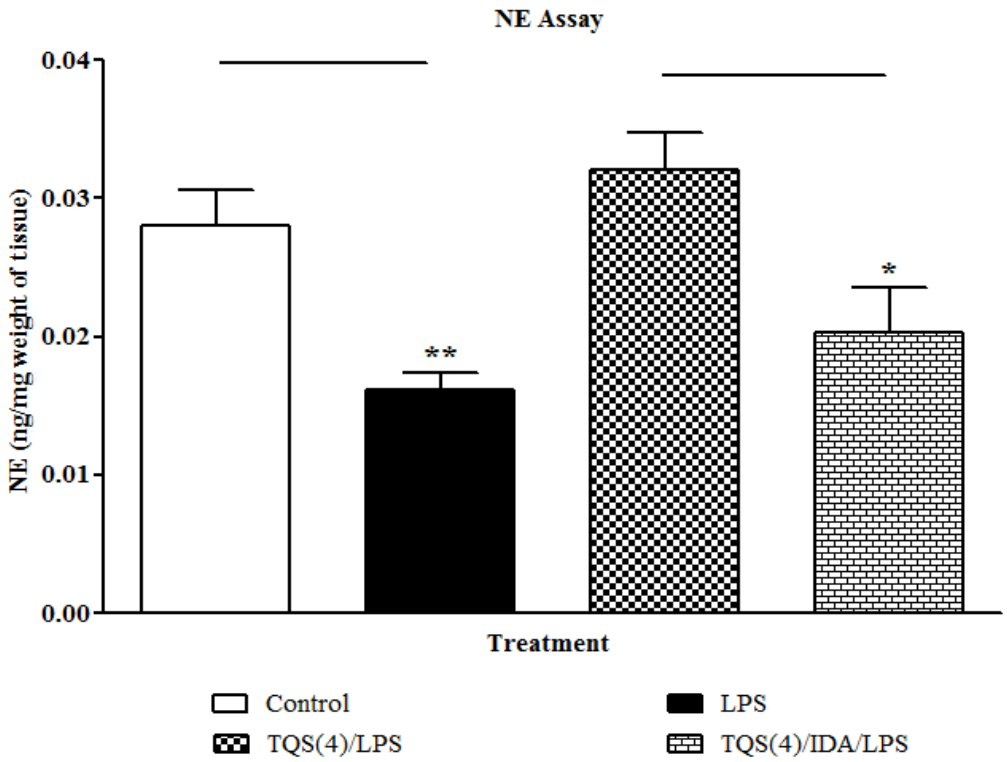

Figure 33: Lipopolysaccharide (LPS) significantly reduced norepinephrine (NE) compared to control. Moreover, idazoxan (IDA) significantly decreased NE compared to TQS treated group. Mice received TQS (4 mg/kg, i.p.) 0.5 h before LPS administration. IDA (2 mg/kg, i.p.) was administered 5 h after LPS administration. Control animals received equal volume of vehicle. Hippocampi were collected 6 h after LPS administration. The NE level was measured by HPLC-EC. Data are expressed as mean ± S.E.M. (n = 5-7/group). *$P < 0.05$; **$P < 0.01$.

Effects of TQS on microglial cells morphology in the hippocampus

The effects of TQS on microglial cells morphology (microglial activation) are displayed in Figure 34. One way ANOVA showed that TQS pretreatment significantly altered microglial activation in the hippocampus ($F_{3,19} = 11.83$; $P < 0.001$). Multiple comparisons of means showed that LPS (1 mg/kg) significantly ($P < 0.001$) elevated microglial activation by increasing microglial morphology in the hippocampus as compared to control. Furthermore, TQS (4 mg/kg) significantly ($P < 0.05$) reduced microglial activation due to reduction in LPS-induced morphological changes in the hippocampus compared to LPS treated group.

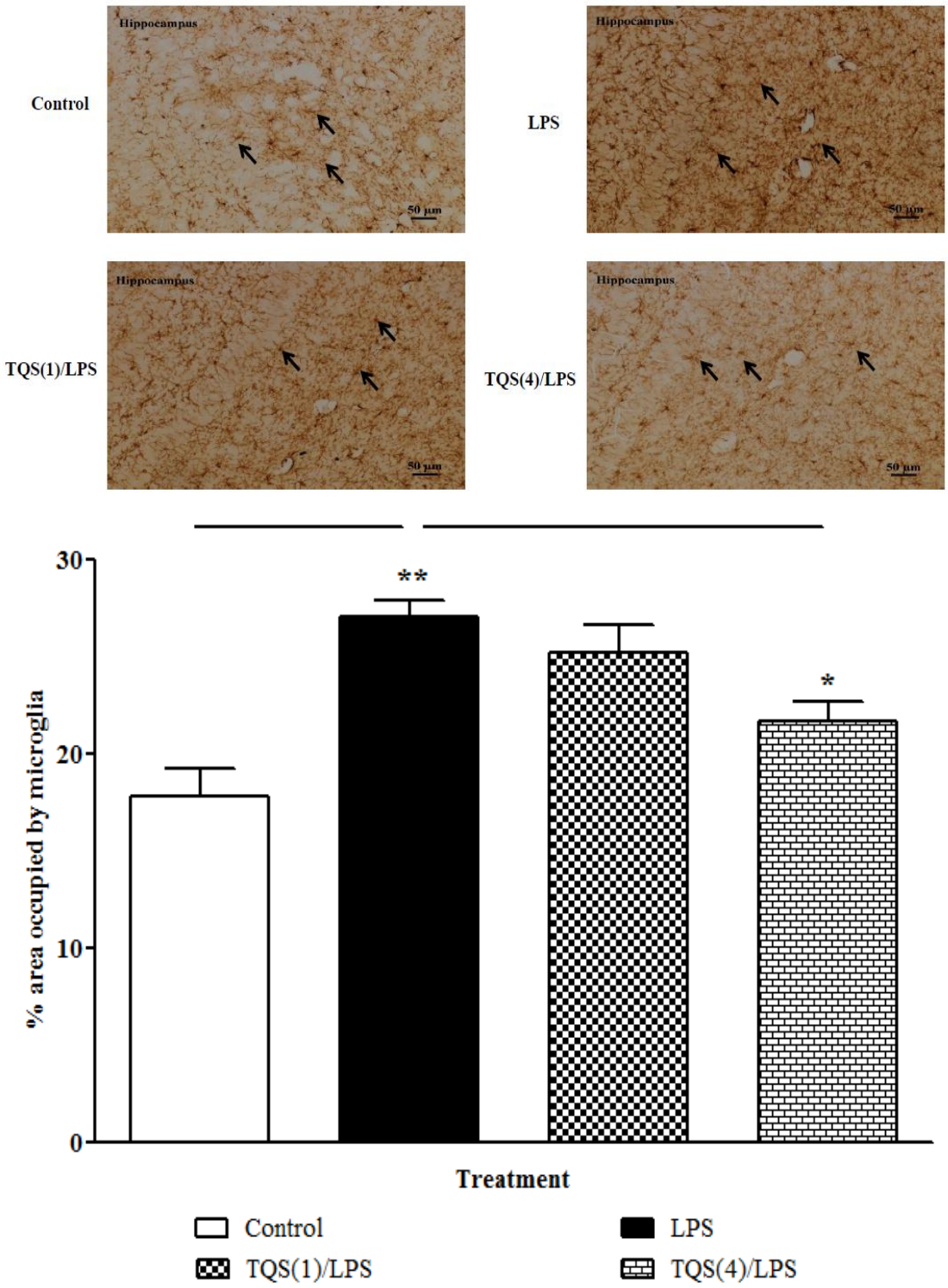
Control
LPS
TQS(1)/LPS
TQS(4)/LPS
Hippocampus
50 µm
30
20
10
0
% area occupied by microglia
**
*
Treatment
Control
LPS
TQS(1)/LPS
TQS(4)/LPS

Figure 34: Lipopolysaccharide (LPS) significantly increased % area occupied by microglial cells (increased microglial activation) compared to control. Moreover, TQS significantly reduced % area occupied by microglial cells (reduced microglial activation) compared to LPS treated group. Animals received TQS (1 or 4 mg/kg, i.p.) 0.5 h before LPS administration. Control animals received equal volume of vehicle. Whole brain was collected 6 h after LPS administration. Microglial morphology was visualized using diaminobenzidine (DAB) immunohistochemistry. Data are expressed as mean $\pm$ SEM. Representative Images indicate microglial morphology in the hippocampus (top panel). Magnification 20 X, Scale bar = 50 um. (n = 5-6/group). *$P < 0.05$; **$P < 0.001$.

4.4. Discussion

The present study demonstrates that administration of TQS increased TNF expression in the hippocampus. Moreover, MLA, an α7 nAChR antagonist, reversed TQS-induced reduction in the expression of hippocampal TNF indicating the involvement of α7 nAChR in TQS-mediated antiallodynic and antihyperalgesic effects. TQS also decreased the immunoreactivity of TNF in CA1 and DG regions of hippocampus suggesting that TQS effects are due to reduced TNF expression in these regions of hippocampus. Furthermore, administration of TQS reduced the level of NE in the hippocampus. TQS at same dose reduced neuroinflammatory pain-like symptoms as reported previously (Abbas and Rahman, 2016). Pretreatment of IDA, an α2 adrenoceptors antagonist, reversed TQS-mediated antiallodynic and antihyperalgesic effects and TQS-induced elevated level of hippocampal NE suggestive TQS decreases neuroinflammatory pain by increasing NE level in the hippocampus. The TQS also reduced LPS-induced morphological changes in the hippocampus indicating TQS

decreases neuroinflammatory pain by reducing microglial activation in the hippocampus. Taken together, our results show that TQS decreases neuroinflammatory pain through decreased TNF expression and enhanced level of hippocampal NE involving α7 nAChR.

Our data support the notion that TNF plays a vital role in neuroinflammatory pain-like symptoms. Elevated level of TNF was previously reported in the hippocampus in neuroinflammatory pain-like symptoms (Covey et al., 2000; Ignatowski et al., 1999; Spengler et al., 2007; Xie et al., 2006). Previous studies show that increased TNF in the hippocampus produces pronociceptive effects. For example, a previous study reported that increasing TNF solely in the hippocampus results in pain hypersensitivity (Martuscello et al., 2012). Furthermore, i.c.v. injection of TNF causes pain hypersensitivity (Oka et al., 1996). Moreover, administration of SiRNA against TNF causes reduction in neuroinflammatory pain-like symptoms in animal models (Gerard et al., 2015). Furthermore, i.c.v. microinfusion of TNF-antibodies reduces neuroinflammatory pain-like symptoms (Ignatowski et al., 1999).

Our results show that TQS distinctly reduces TNF expression in the hippocampus. This showed that TQS-induces antiallodynic and antihyperalgesic effects against neuroinflammatory pain are due to reduced TNF in the hippocampus. Moreover, MLA reversed TQS-mediated decrease in the expression of TNF mRNA expression in the hippocampus indicating reduction in TNF expression is linked with α7 nAChR. Furthermore, TQS reduced the immunoreactivity of TNF both in DG and CA1 regions of hippocampus. Therefore, we consider that TQS decreases neuroinflammatory pain by reducing TNF in these two hippocampal regions. Moreover, LPS did not elevate TNF expression in the brain stem (results not shown) suggesting TNF increases only in the

higher brain centers, including hippocampus. One probable reason may be due to high density of microglial cells in hippocampus as compared to other brain regions (Lawson et al., 1990) that may result in increased microglial activation as reported previously (Silverman et al., 2014) and increased TNF expression following LPS administration.

The effects of α7 nAChRs in the reduction of neuroinflammation have been well documented. However, exact mechanism by which these ligands reduce neuroinflammation involving α7 nAChRs is not clearly understood. Moreover, earlier studies showed that microglial α7 nAChRs are metabotropic receptors, it is, therefore probable that TQS decreases neuroinflammatory pain-like symptoms by reducing the expression of TNF through downstream signaling mediators involving NF-κB inactivation and microglial α7 nAChR in the hippocampus.

Previous studies showed that TNF is critically involved in NE release in the hippocampus. For example, a study showed that NE level decreases in the hippocampus due to elevated TNF in increased painful conditions. Moreover, NE level increases in the hippocampus due to reduced expression of TNF. Our results showed that LPS decreases NE level in pain hypersensitivity. This may be correlated with elevated TNF as described previously. Furthermore, IDA reversed the TQS-mediated increase in NE release suggesting α2 adrenoceptors involvement in the release of NE. Taken together these results indicate that TQS mediate its antiallodynic and antihyperalgesic effects involving increased release of NE and reduced expression of TNF in the hippocampus.

The mechanism through which the release of NE is controlled in the hippocampus involves stimulation of α2-adrenoceptors with NE at presynaptic level of adrenergic neurons (Dixon et al., 1979). Moreover, cytokines and neurotransmitters are signals of

foremost importance that play very important role in bidirectional communication between immune systems and neurons thus maintaining homeostasis. The abnormal production of one or both of these markers greatly affects the signaling associated by the other marker leading to pathology. For example, a previous study demonstrated that TNF plays a very critical role in the release of NE from adrenergic neurons. However, the exact mechanism through which TNF reduces NE release in the hippocampus is not clearly known.

Previous study indicated that during pain hypersensitivity, hippocampal NE level is decreased (Covey et al., 2000) due to elevated TNF. This reduction in NE is linked with dysregulation of G-protein coupling with presynaptic α2-adrenergic receptor-associated with elevated TNF (Ignatowski and Spengler, 1994; Millan, 2002; Romanelli and Esposito, 2004; Yaksh, 1985). Therefore, an interaction exits between TNF expression and G-protein coupling with α2-adrenoceptors affecting the release of NE in the hippocampus. For example, it was shown that elevated expression of TNF remodels Gi coupling more than Gs with α2-adrenoceptors causing decrease release of NE in painful conditions (Covey et al., 2000; Ignatowski et al., 1999).

Our results showed that LPS caused increased TNF expression and reduced NE level suggesting coupling of Gi more than Gs with α2-adrenergic receptors. TQS pretreatment on the other hand caused in reduction in the expression of TNF and increased NE level in the hippocampus demonstrating Gs coupling more than Gi with α2-adrenoceptors. Amitriptyline, a tricyclic antidepressant, used in the treatment of painful conditions has been hypothesized to work same mechanism involving decreased release of TNF and increased release of NE in the hippocampus (Ignatowski et al., 2005).

However, further studies are warranted involving the measurement of Gi and Gs level of hippocampal noradrenergic neurons in LPS-induced neuroinflammatory pain. Taken together our data indicate that TQS decreased neuroinflammatory pain due to reduced TNF expression and increased NE level in the hippocampus.

The TLR4 is a pattern recognition receptor that binds to LPS and activates signaling pathway that causes NF-κB and microglial activation (Palsson-McDermott and O'Neill, 2004; Rivest, 2003). Microglial activation is determined by measurement of elevated expression of Iba-1 which is restricted to microglia (Abbas and Rahman, 2016). Furthermore, microglial cells adopt resting phenotype in inactivated state but undergo rapid activation in response to acute insults. This results in change in their morphological phenotype characterized by retracted processes, hypertrophy and amoeboid morphology under strongly pathological conditions (Chen et al., 2012; Tsuda et al., 2005; Watkins and Maier, 2003; Zielasek and Hartung, 1996). Our results show that TQS-mediated antiallodynic and antihyperalgesic effects are due to reduced microglial activation as indicated by reduced morphological changes in the hippocampus.

In conclusion, our results show that LPS-induced tactile allodynia and thermal hyperalgesia are due to elevated TNF and reduced NE level in the hippocampus. Moreover, our study demonstrated that microglial α7 nAChR positive allosteric modulation in the hippocampus is involved in reducing neuroinflammatory pain-like symptoms involving decreased TNF expression and increased NE level. Overall, we propose that reduction in TNF expression and elevated level of NE in the hippocampal is a critical and attractive therapeutic approach for the clinical utility of neuroinflammatory pain.

Chapter 5: Effects of α7 nAChR PAM on microglial BDNF expression in the hippocampus

5.1. Introduction

We have found in chapter 3 that activation of microglial α7 nAChR results in reduced NF-κB activation in neuroinflammatory pain. The active NF-κB was shown to increase BDNF expression from microglial cells (Ji and Suter, 2007). The BDNF has been reported to be distributed in whole brain with highest level in the hippocampus (Ernfors et al., 1990). Unlike proinflammatory cytokines, BDNF is not regarded as inflammatory mediator. However, it plays very important role in the development and maintenance of neuroinflammatory pain (Coull et al., 2005; Groth and Aanonsen, 2002; Mannion et al., 1999; Pezet and Malcangio, 2004). The levels of BDNF proteins and mRNA are increased after neuroinflammation (Mannion et al., 1999). Moreover, LPS is known to increase the expression of BDNF from microglial cells (Gomes et al., 2013; Miwa et al., 1997; Tomaz et al., 2014).

The BDNF was found to induce pain hypersensitivity at 4 h after administration (Guo et al., 2006). The neuroinflammatory pain-like symptoms are decreased by preventing the release of BDNF from microglia (Coull et al., 2005). Moreover, microinjection of BDNF in CNS causes increased pain behavior in animal models (Zhou et al., 2000). The BDNF mediates its effects through TrkB and subsequent activation signaling cascade(Huang and Reichardt, 2003). The neuroinflammatory pain-like symptoms are decreased by blocking the signaling between BDNF and TrkB (Coull et al., 2005). Moreover, supraspinal BDNF-TrkB signaling cascade favors pain facilitation (Guo et al., 2006). Therefore, BDNF-TrkB signaling pathway represents novel

mechanism for pain facilitation in LPS-induced neuroinflammatory pain model and blocking this microglia-neuron signaling pathway might be novel strategy for the treatment of neuroinflammatory pain.

We have previously reported that TQS, decreases neuroinflammatory pain due to reduced microglial and NF-κB activation in the hippocampus. However, the effects of α7 nAChR PAMs on BDNF expression in the hippocampus are not known yet. We, therefore, hypothesized that targeting microglial BDNF within hippocampus via α7 nAChRs PAM will have potential therapeutic ability against neuroinflammatory pain.

The ANA-12 is a selective, potent, and noncompetitive TrkB antagonist (Cazorla et al., 2011). It has been shown to cross BBB after systemic administration. The active concentration of ANA-12 was detected in the brain as soon as 30 minutes and remain upto 6 hours (Cazorla et al., 2011). The ANA-12 is used to examine the role of BDNF at TrkB (Zhang et al., 2015).

As indicated in chapter 3, the CA1 and DG regions of the hippocampus are critically involved in pain production and maintenance. These regions of the hippocampus are densely populated with microglial cells compared with most of other brain regions indicating potential role of microglial cells in these regions in neuroinflammatory pain involving BDNF. We, therefore, hypothesized that TQS will reduce microglial BDNF expression in the hippocampus during antiallodynic and antihyperalgesic effects as reported previously. In current study, we have determined the effects of TQS on BDNF expression in the hippocampus. In addition, we have examined the effects of TQS on Iba-1 expression in the hippocampus in mice.

5.2. Methods

Animals

Male C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed four in each cage under standard laboratory conditions as described before (see chapter 2, section 2.2).

Induction of tactile allodynia and thermal hyperalgesia

Tactile allodynia and thermal hyperalgesia were induced as mentioned earlier (see chapter 2, section 2.2). ANA-12 (0.25 or 0.5 mg/kg) was reconstituted in normal saline having 0.5% tween 80 and 3.5% DMSO and was given 10 min before TQS injection. Control animals received an equal volume of vehicle. The source, method of reconstitution, and time of administration for other drugs used in current study mentioned earlier (see chapter 2, section 2.2). The doses for TQS, MLA, and ANA-12 were selected based our previous report and others (Abbas and Rahman, 2016; Callahan et al., 2013; Zhang et al., 2015).

Tactile allodynia

Tactile allodynia was measured as described previously (see chapter 2, section 2.2).

Thermal hyperalgesia

Thermal hyperalgesia was quantified as described before (see chapter 2, section 2.2).

RNA isolation and cDNA synthesis

Total RNA was isolated from hippocampal tissue as described earlier (see chapter 3, section 3.2).

Quantitative real-time PCR

Quantitative real-time PCR was performed as mentioned before (see chapter 3, section 3.2). Primers sequences were obtained from Integrated DNA Technologies (Coralville, Iowa, USA) and are displayed in Table 3.

Table 3: Prime primer sequence for GAPDH, and BDNF

Gene	**Primer sequence (5'-3')**
GAPDH	GTGGAGTCATACTGGAACATGTAG (forward)
	AATGGTGAAGGTCGGTGTG (reverse)
BDNF	GCAACCGAAGTATGAAATAACCA (forward)
	GTTTATCACCAGGATCTAGCCA (reverse)

Western blot analysis

Western blot analysis was carried out as described in earlier (see chapter 2, section 2.2) with minor modification. Briefly, mice were sacrificed six h after LPS administration and their hippocampi was dissected out. The brain tissue samples were homogenized in RIPA buffer containing Dulbecco's Phosphate-buffered saline (pH 7.4), 0.1% sodium dodecyl sulfate, 1% Igepal CA-630, and protease inhibitor mix (complete, Mini, Roche, Indianapolis, IN, USA). Equal amounts of protein (50 μg) were loaded onto 12% gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis. Separated proteins were transferred onto immobilon-P transfer membrane (EMD Millipore Corporation, MA, USA) at 40 volt overnight at 4°C. Membranes were then blocked on a

gyro-rocker with 5% nonfat dry milk in Tris-buffered saline/0.1% tween-20 for 50 min, and then incubated overnight at 4°C with primary antibodies for BDNF (H-117, 1:300, rabbit polyclonal, Santa Cruz Biotech, USA), and subsequently with appropriate secondary antibody. The β-tubulin (E7-S, 1:5,000, mouse monoclonal, University of Iowa, USA) was used as loading control.

Immunofluorescence assay

Immunofluorescence was carried out as mentioned before (chapter 3, see section 3.2) with minor modification. Briefly, primary antibody for BDNF (H-117, 1:300, rabbit polyclonal, Santa Cruz Biotech, USA) and secondary antibody labeled with FITC (1:50) (Santa Cruz Biotechnology, Dallas, TX, USA) were used. Images were captured using a laser scanning confocal microscope (Olympus Fluoview FV1200).

Statistical analyses

For thermal hyperalgesia, Western blot analysis, immunofluorescence assay, and quantitative real-time PCR, data were analyzed by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. For quantitative real-time PCR for MLA and TQS/ANA-12 combination data for thermal hyperalgesia, Student's t-test was used. For tactile allodynia, Kruskil-Wallis test followed by Dunn's post hoc test for multiple comparisons or Mann-Whitney test for TQS/ANA-12 combination was applied using GraphPad Prism 5.0 (GraphPad Inc., San Diego, CA, USA). The difference between treatments was considered as significant at $P < 0.05$. All results are expressed as mean ± S.E.M.

5.3. Results

Effects of ANA-12 on 50% paw withdrawal in von Fey filament test in LPS-induced neuroinflammatory pain

The effects of ANA-12 on 50% paw withdrawal (tactile allodynia) in von Frey filament test are shown in Figure 35. Kruskal-wallis test showed that ANA-12 significantly decreased 50% paw withdrawal threshold ($F_{3,21} = 26.67$; $P < 0.0001$). Multiple comparisons of means revealed that LPS (1 mg/kg) significantly ($P < 0.001$) decreased 50% paw withdrawal threshold compared to control showing the presence of pain hypersensitivity. Furthermore, ANA-12 (0.5 mg/kg) significantly ($P < 0.01$) enhanced 50% paw withdrawal threshold compared to LPS treatment group indicating antiallodynic effects of ANA-12 are due to the blockade of BDNF effects at TrkB.

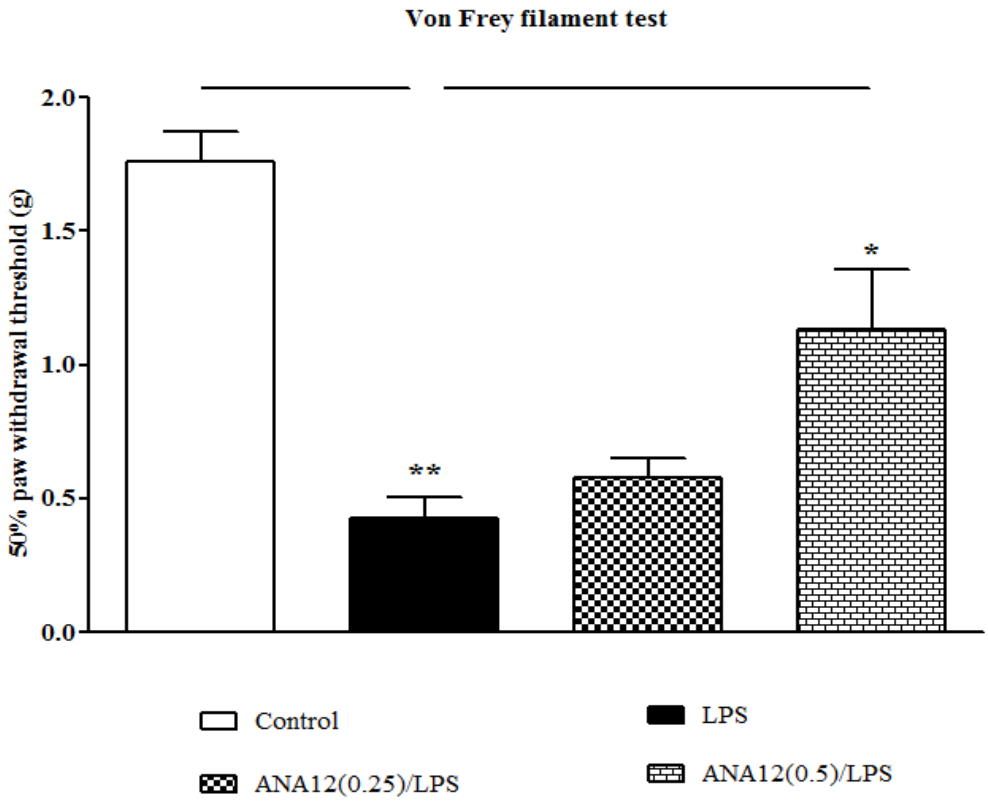

Figure 35: Lipopolysaccharide (LPS) significantly decreased 50% paw withdrawal threshold (tactile allodynia) compared to control. Moreover, ANA-12 significantly increased 50% paw withdrawal threshold compared to LPS treated group. Mice received ANA-12 (0.25 or 0.5 mg/kg, i.p.) 30 min before LPS administration. Control animals received equal volume of vehicle. Paw withdrawal threshold was assessed using von Frey filaments six h after LPS administration. Data are expressed as mean $\pm$ S.E.M. (n = 5-8/group). $^{*}P < 0.01$, $^{**}P < 0.001$.

Effects of ANA-12 on latency time in hot plate test in LPS-induced neuroinflammatory pain

The effects of ANA-12 on latency time (thermal hyperalgesia) in hot plate test are shown in Figure 36. One way ANOVA showed that ANA-12 significantly altered latency time in hot plate test ($F_{3,19} = 8.774$; $P < 0.001$). Multiple comparisons of means showed that LPS (1 mg/kg) significantly ($P < 0.001$) reduced hot plate latency as compared to control group indicating significant reduction in hot plate latency time. Moreover, ANA-12 (0.5 mg/kg) significantly ($P < 0.05$) enhanced hot plate latency compared to LPS treatment group indicating antihyperalgesic effects of ANA-12 are due to the blockade of BDNF effects at TrkB.

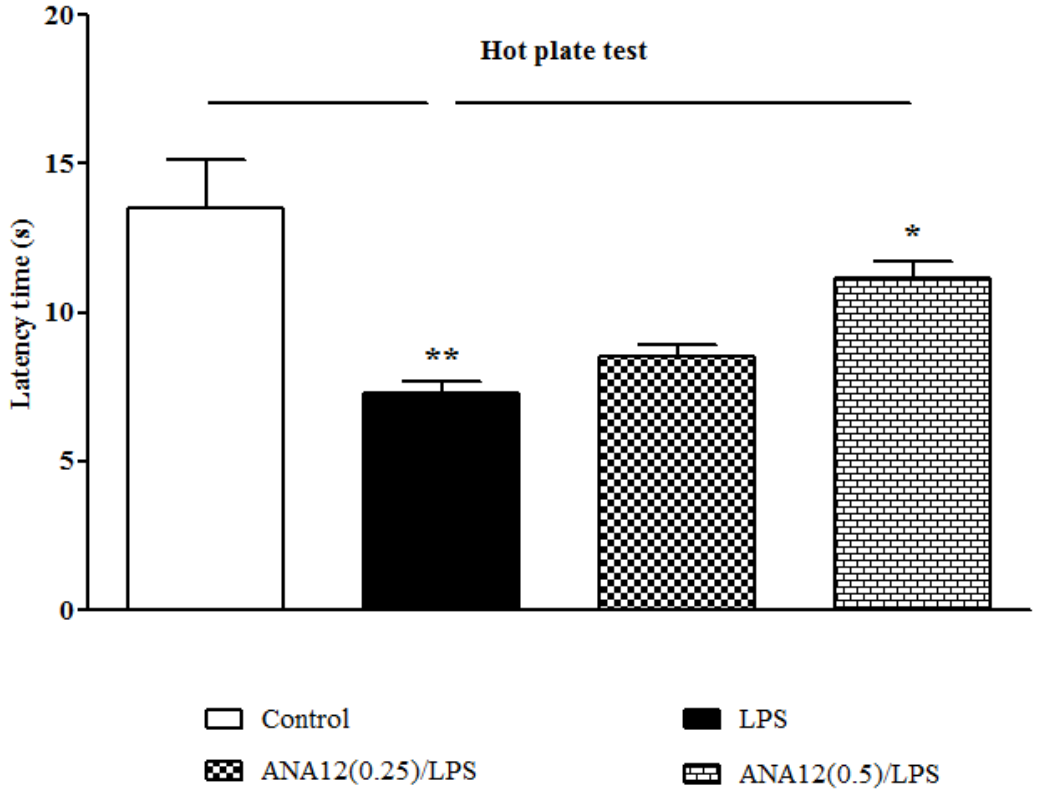

Figure 36: Lipopolysaccharide (LPS) significantly decreased latency time (thermal hyperalgesia) on the hot plate. Moreover, ANA-12 significantly increased latency time on hot plate compared to LPS treated group. Mice received ANA-12 (0.25 or 0.5 mg/kg, i.p.) 30 min before LPS administration. Control animals received equal volume of vehicle. Latency on hot plate was measured six h after LPS administration. Data are expressed as mean ± S.E.M. (n = 5-6/group).*$P < 0.05$; **$P < 0.001$.

Effects of co-administration of TQS and ANA-12 on 50% paw withdrawal threshold in von Fey filament test in LPS-induced neuroinflammatory pain

The effects of co-administration of TQS and ANA-12 on 50% paw withdrawal threshold (tactile allodynia) are displayed in Figure 37. Mann-Whitney test showed that co-administration of TQS and ANA-12 significantly ($P < 0.05$) decreased 50% paw

withdrawal threshold indicating reduction in the expression of BDNF during TQS-mediated antiallodynic response.

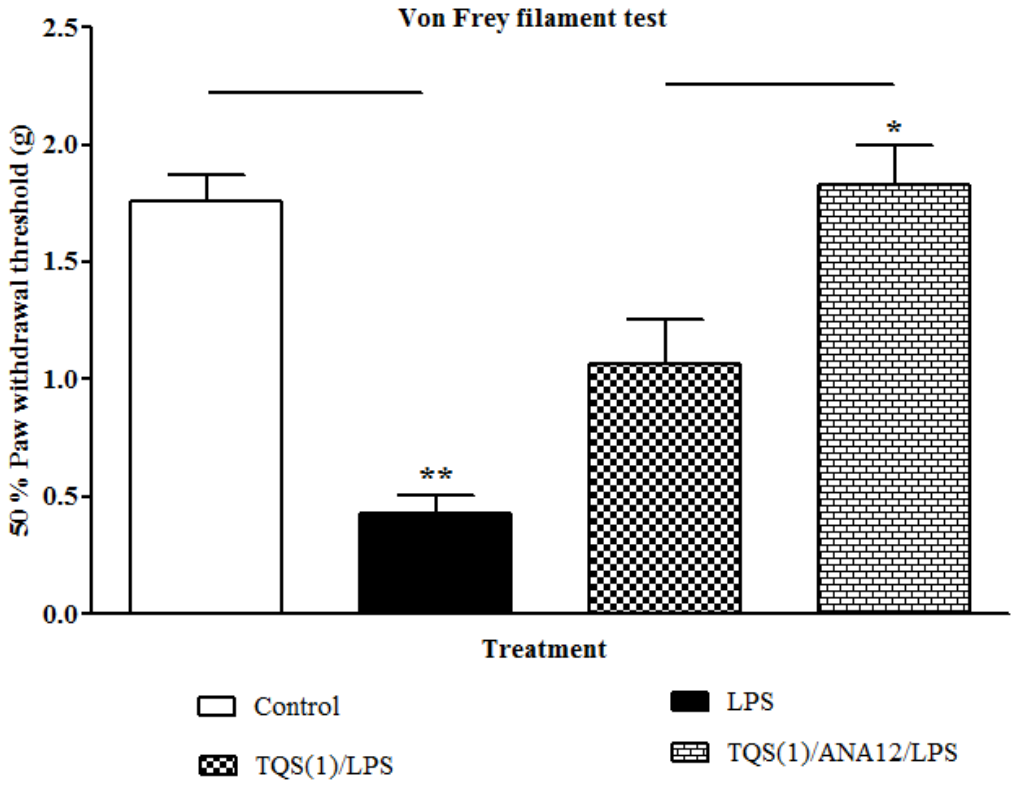

Figure 37: Lipopolysaccharide (LPS) significantly decreased 50% paw withdrawal threshold (tactile allodynia) compared to control. Furthermore, TQS in combination with ANA-12 significantly increased 50% paw withdrawal threshold compared to TQS treated group. Mice received ANA-12 (0.25 mg/kg, i.p.) 10 minutes before TQS (1 mg/kg, i.p.) injection. The TQS was given 30 minutes before LPS administration. Control animals received equal volume of vehicle. Paw withdrawal threshold was assessed using von Frey filaments six h after LPS administration. Data are expressed as mean $\pm$ S.E.M. (n = 5-8 group). $^{*}P < 0.05$, $^{**}P < 0.001$.

Effects of co-administration of TQS and ANA-12 on latency time in hot plate test in LPS-induced neuroinflammatory pain

The effects of co-administration of TQS and ANA-12 on latency time (thermal hyperalgesia) are displayed in Figure 38. Student's t-test revealed that co-administration of TQS and ANA-12 significantly ($P < 0.0001$) elevated latency time in hot plate test indicating antihyperalgesic effects of TQS are mediated through reduced BDNF expression.

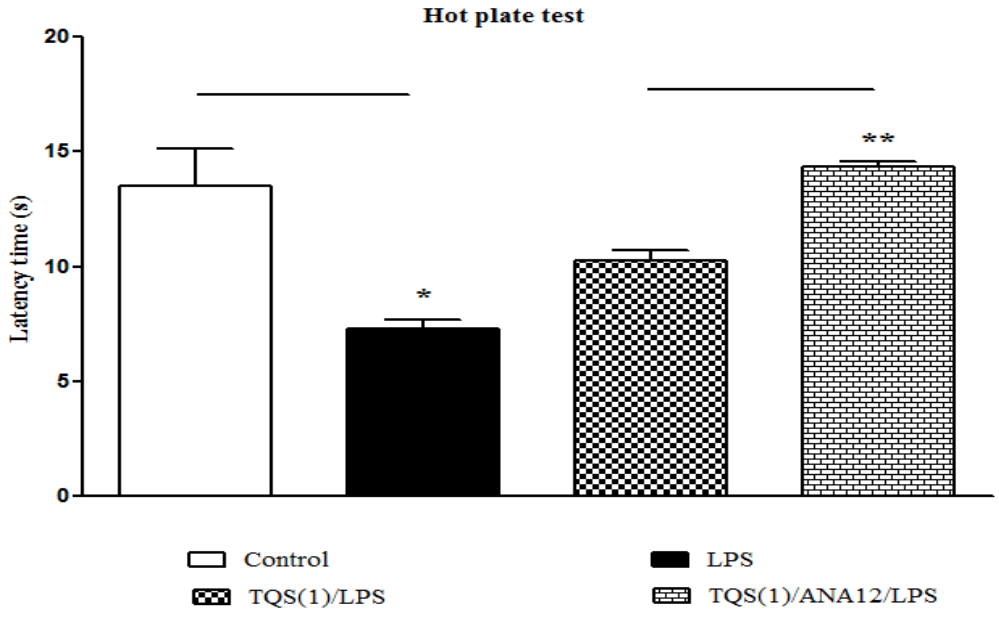

Figure 38: Lipopolysaccharide (LPS) significantly decreased latency time (thermal hyperalgesia) on the hot plate. Moreover, TQS in combination with ANA-12 significantly increased latency time on hot plate compared to TQS treated group. Mice received ANA-12 (0.25 mg/kg, i.p.) 10 minutes before TQS (1 mg/kg, i.p.) injection. TQS was given 0.5 h before LPS administration. Control animals received equal volume of vehicle. Latency on hot plate was measured six h after LPS administration. Data are expressed as mean $\pm$ S.E.M. (n = 5-6/group). *$P < 0.01$; **$P < 0.001$.

Effects of TQS on BDNF mRNA expression in the hippocampus

The effects of TQS on the expression of BDNF mRNA are displayed in Figure 39. One way ANOVA showed that TQS significantly reduced the expression of BDNF mRNA ($F_{3,13} = 8.128$; $P < 0.01$). Multiple comparisons of means showed that LPS (1 mg/kg) significantly ($P < 0.05$) elevated the expression of BDNF mRNA suggesting LPS-induced neuroinflammatory pain is due to increased expression of BDNF. Furthermore, TQS (1 or 4 mg/kg) significantly ($P < 0.01$) reduced the expression of BDNF mRNA in LPS-induced neuroinflammatory pain supporting the hypothesis that TQS reduced neuroinflammatory pain-like symptoms by reducing BDNF expression in the hippocampus.

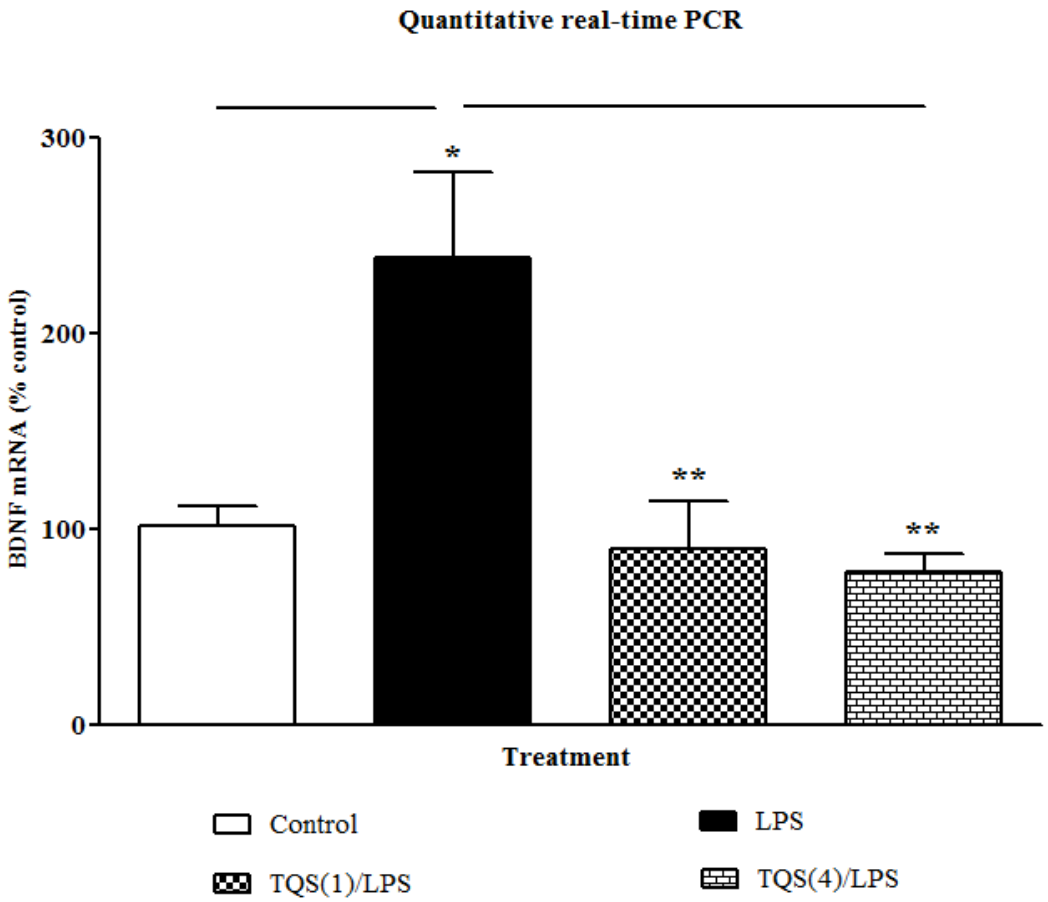

Figure 39: Lipopolysaccharide (LPS) significantly increased brain-derived neurotrophic factor (BDNF) mRNA expression compared to control. Moreover, TQS significantly reduced the expression of BDNF mRNA compared to LPS treated group. Animals were administered TQS (1 or 4 mg/kg, i.p.) 0.5 h before LPS administration. Control animals received equal volume of vehicle. Hippocampi were collected 6 h after LPS administration. The BDNF mRNA expression was quantified using quantitative real-time polymerase chain reaction. Data are expressed as mean $\pm$ S.E.M. (n = 4-5/group). *P < 0.05, **P < 0.01.

Effects of MLA on TQS-induced decrease in BDNF mRNA expression in the hippocampus

The effects of MLA on TQS-induced decrease in BDNF mRNA expression are displayed in Figure 40. Student's t-test indicated that MLA significantly (P < 0.01) reversed TQS-induced decrease in BDNF mRNA expression. This confirmed that TQS-induced decrease in BDNF mRNA is mediated through α7 nAChR.

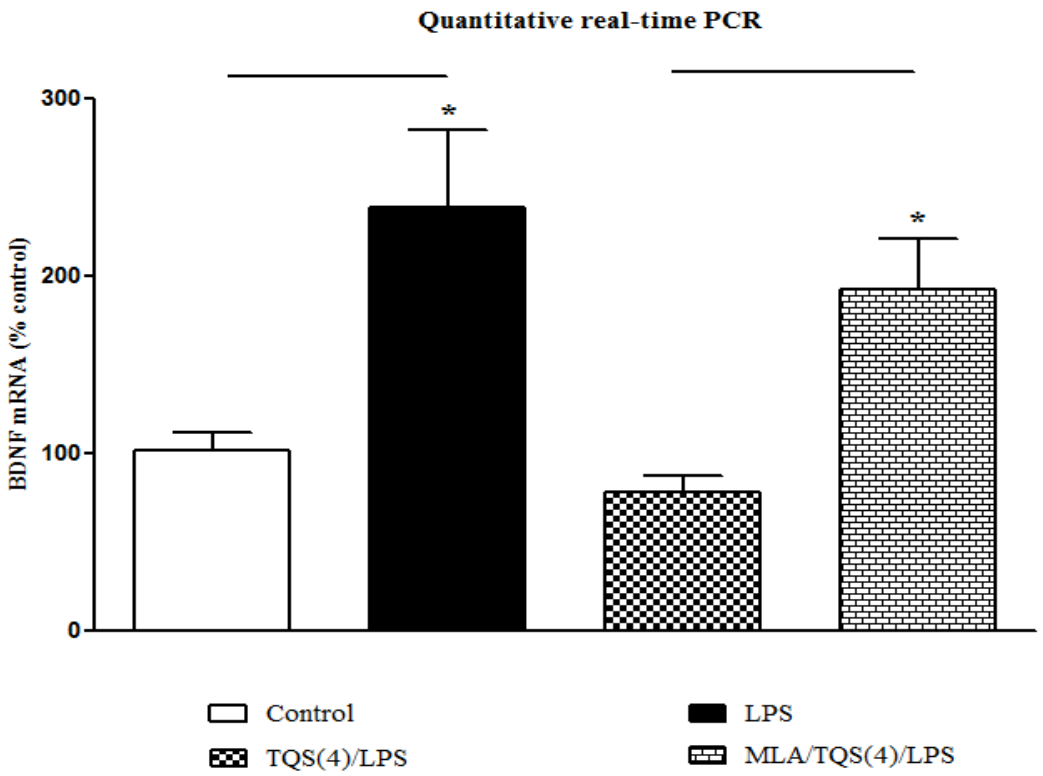

Figure 40: Lipolpolysaccharide (LPS) significantly increased brain-derived neurotrophic factor **(**BDNF**)** mRNA expression compared to control. Methyllycaconitine (MLA) significantly increased BDNF mRNA expression compared to TQS treatment group. Mice were administered TQS (4 mg /kg, i.p.) 0.5 h before LPS administration. The MLA (3 mg /kg, i.p.) was given 10 min before TQS injection. Control animals received equal volume of vehicle. Hippocampi were collected 6 h after LPS administration. The BDNF mRNA expression was quantified using quantitative real-time polymerase chain reaction. Data are expressed as mean $\pm$ S.E.M. (n = 4-5/group). *$P < 0.05$; **$P < 0.01$.

Effects of TQS on BDNF expression in the hippocampus

The effects of TQS on BDNF expression are displayed in Figure 41. One way ANOVA showed that TQS significantly reduced the expression of BDNF ($F_{3,12} = 5.204$; $P < 0.05$). Multiple comparisons of means showed that LPS (1 mg/kg) significantly ($P <$

0.05) elevated BDNF expression suggesting LPS-induced neuroinflammatory pain is due to increased BDNF expression in the hippocampus. Furthermore, TQS (4 mg/kg) significantly ($P < 0.05$) reduced BDNF expression in LPS-induced neuroinflammatory pain supporting our hypothesis that TQS decreased neuroinflammatory pain-like symptoms by decreasing BDNF expression in the hippocampus.

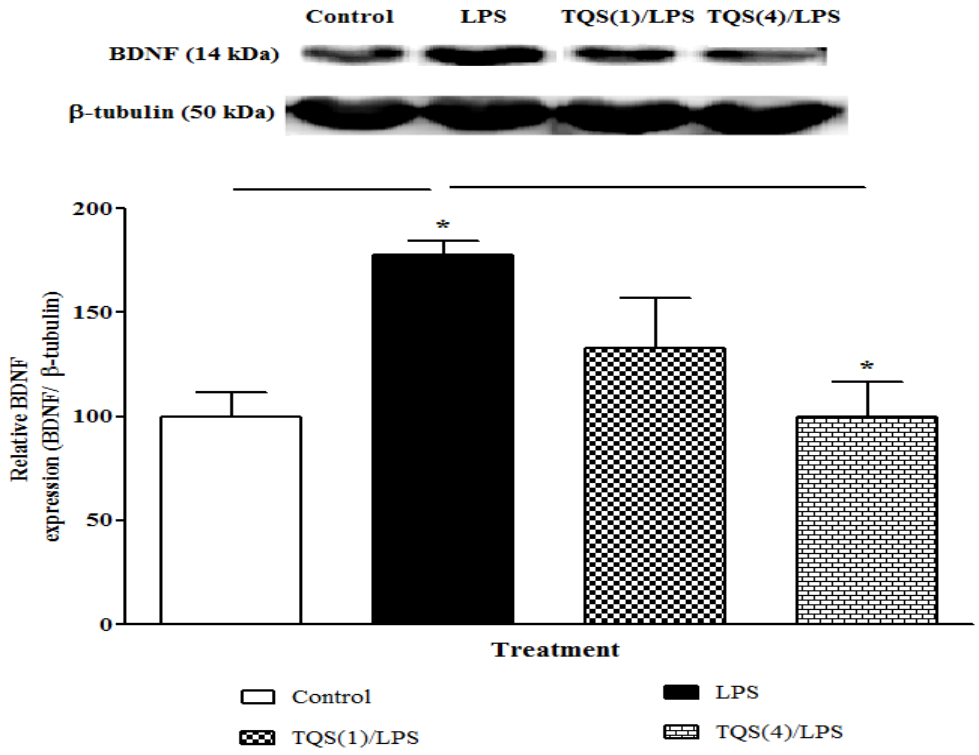

Figure 41: Lipopolysaccharide (LPS) significantly increased brain-derived neurotrophic factor (BDNF) expression compared to control. Moreover, TQS significantly reduced the expression of BDNF compared to LPS treated group. Animals were administered TQS (1 or 4 mg/kg, i.p.) 0.5 h before LPS administration. Control animals received equal volume of vehicle. Hippocampi were collected 6 h after LPS administration. Representative Western blots for BDNF expression from hippocampus (top panel). Data are expressed as mean $\pm$ S.E.M. (n = 4/group) *$P < 0.05$.

Effects of TQS on BDNF expression in CA1 in hippocampus

The effects of TQS on BDNF expression in CA1 region of the hippocampus are displayed in Figure 42. One way ANOVA showed that TQS significantly decreased BDNF expression in LPS-induced neuroinflammatory pain ($F_{2,11} = 22.39$; $P < 0.001$). Multiple comparisons of means showed that LPS (1 mg/kg) significantly ($P < 0.001$) elevated the expression of BDNF. Furthermore, TQS (4 mg/kg) significantly ($P < 0.001$) decreased BDNF expression in CA1 region of hippocampus.

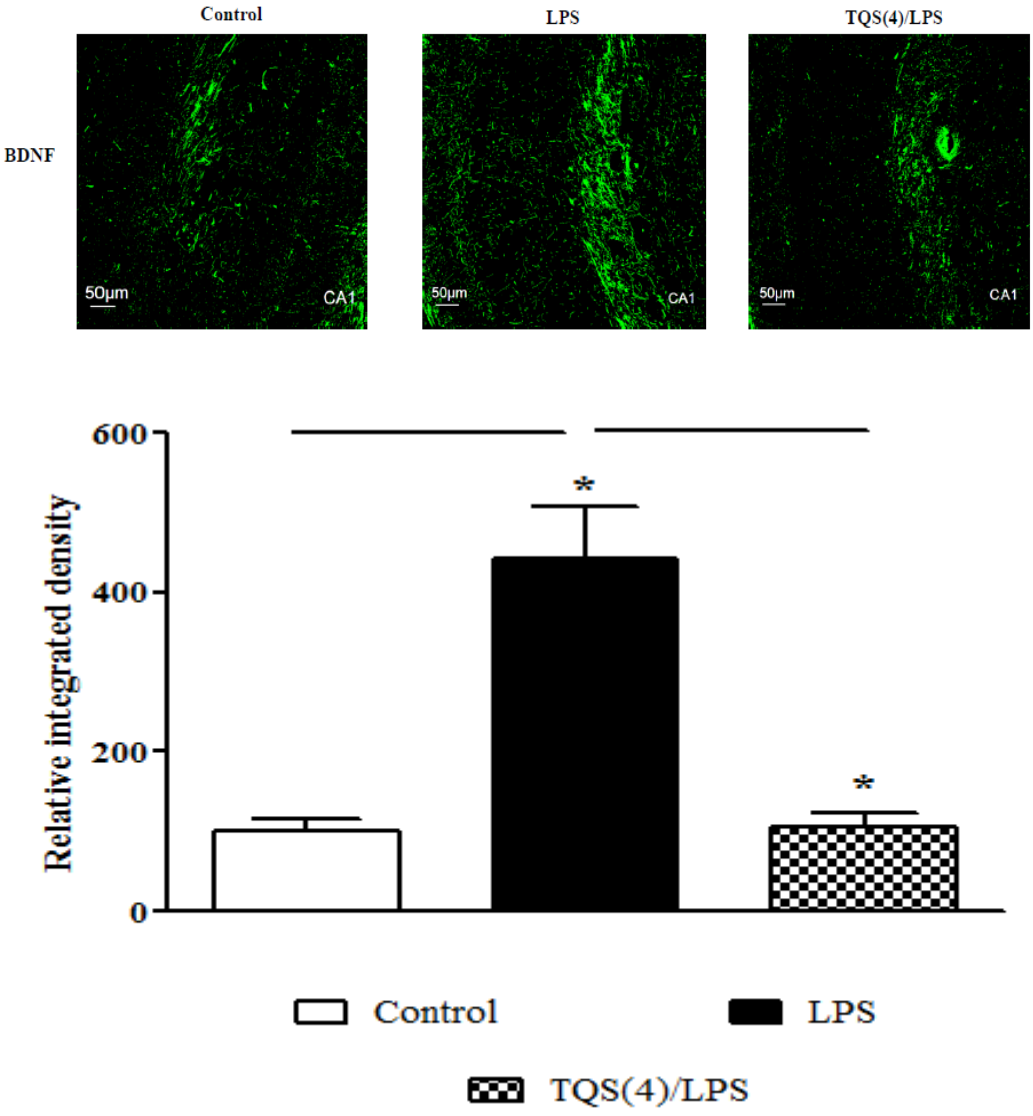

Figure 42: Lipopolysaccharide (LPS) significantly increased brain-derived neurotrophic factor (BDNF) expression in CA1 region of hippocampus compared to control. Moreover, TQS significantly reduced the expression of BDNF compared to LPS treated group. Mice were administered TQS (4 mg/kg, i.p.) 0.5 h before LPS administration. Control mice received equal volume of vehicle. Whole brain was collected 6 h after LPS administration. The BDNF expression was quantified using immunofluorescence assay. Data are expressed as mean $\pm$ S.E.M. Representative images show the immunofluorescence in CA1 in hippocampus. Magnification 20 X, Scale bar = 50 um. (n = 4-5/group). *$P < 0.001$.

Effects of TQS on BDNF expression in DG in hippocampus

The effects of TQS on BDNF expression in DG region of the hippocampus are displayed in Figure 43. One way ANOVA indicated that TQS significantly decreased the expression of BDNF in LPS-induced neuroinflammatory pain ($F_{2,12} = 41.70$; $P < 0.0001$). Multiple comparisons of means showed that LPS (1 mg/kg) significantly ($P < 0.001$) elevated the expression of BDNF. Furthermore, TQS (4 mg/kg) significantly ($P < 0.001$) decreased BDNF expression in DG region of hippocampus.

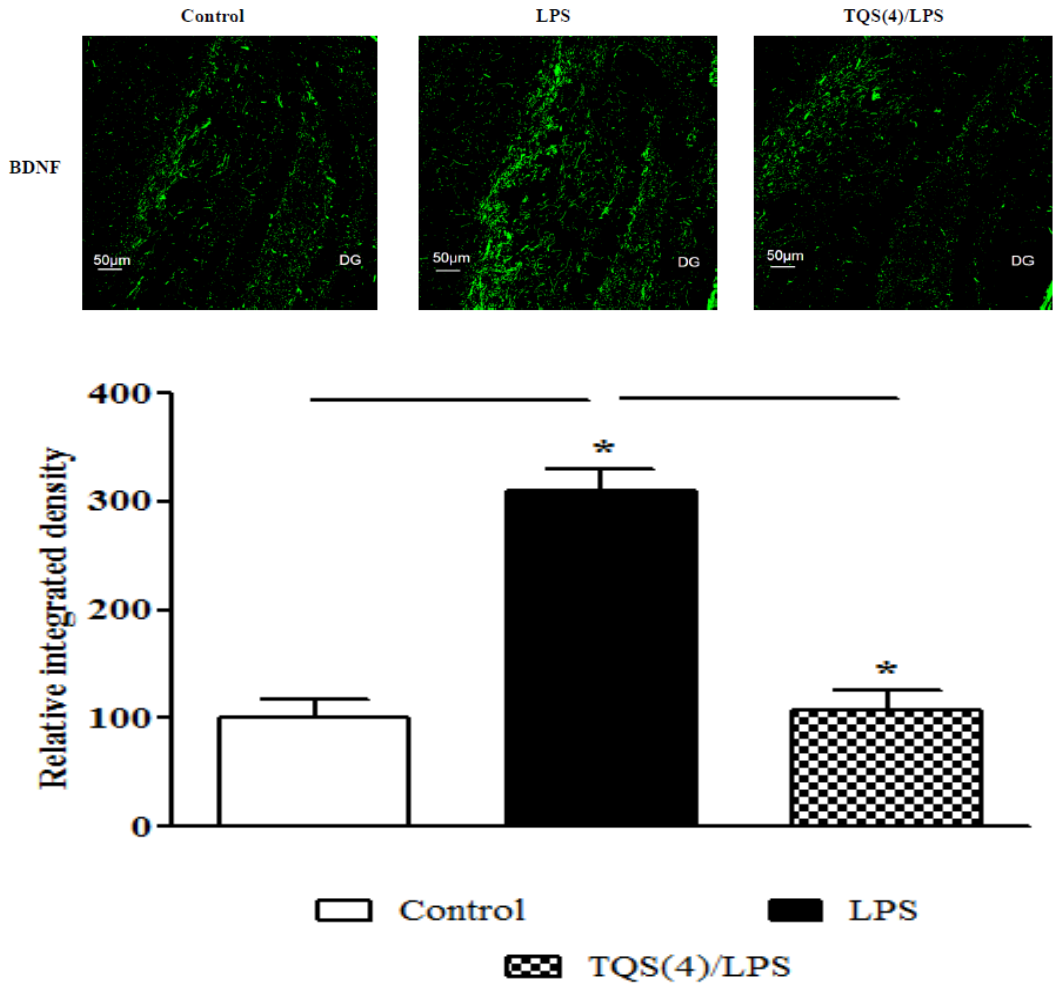

Figure 43: Lipopolysaccharide (LPS) significantly increased brain-derived neurotrophic factor (BDNF) expression in dentate gyrus (DG) of hippocampus compared to control. Moreover, TQS significantly reduced the expression of BDNF compared to LPS treated group. Mice were administered TQS (4 mg/kg, i.p.) 0.5 h before LPS administration. Control mice received equal volume of vehicle. Whole brain was collected 6 h after LPS administration. The BDNF expression was quantified using immunofluorescence assay. Data are expressed as mean $\pm$ S.E.M. Representative images show the immunofluorescence in DG in hippocampus. Magnification 20 X, Scale bar = 50 um. (n = 5/group). *$P < 0.001$.

Effects of TQS on Iba-1 expression in CA1 in hippocampus

The effects of TQS on Iba-1 expression in CA1 region of the hippocampus are displayed in Figure 44. One way ANOVA showed that TQS significantly affected Iba-1 expression in LPS-induced neuroinflammatory pain ($F_{2,9} = 18.34$; $P < 0.001$). Multiple comparisons of means showed that LPS (1 mg/kg) significantly ($P < 0.01$) elevated the expression of Iba-1. Furthermore, TQS (4 mg/kg) significantly ($P < 0.01$) decreased Iba-1 expression in CA1 region of hippocampus.

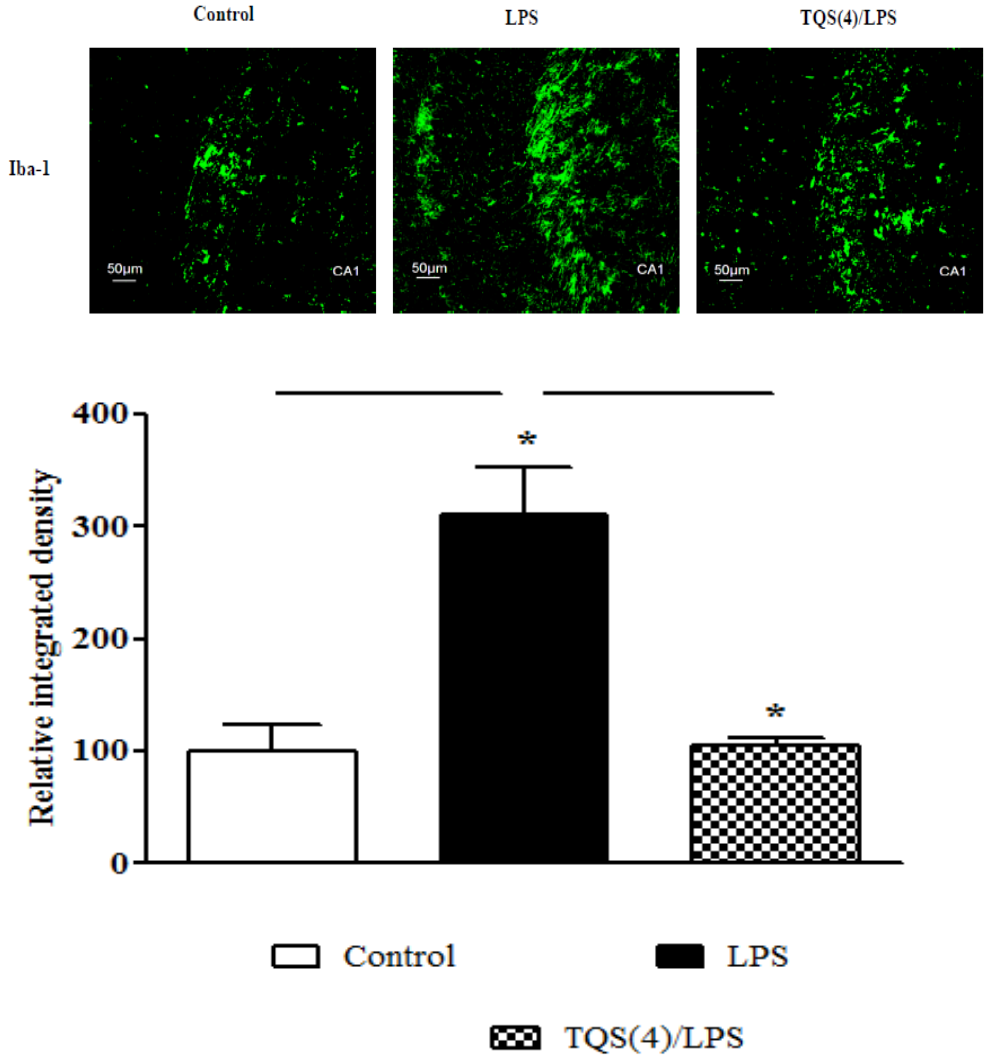

Figure 44: Lipopolysaccharide (LPS) significantly increased ionized-calcium binding adaptor molecule 1 (Iba-1) expression compared to control in CA1 region of hippocampus. Moreover, TQS significantly reduced the expression of Iba-1 compared to LPS treated group. Mice were administered TQS (4 mg/kg, i.p.) 0.5 h before LPS administration. Control mice received equal volume of vehicle. Whole brain was collected 6 h after LPS administration. The Iba-1 expression was quantified using immunofluorescence assay. Data are expressed as mean $\pm$ S.E.M. Representative images show the immunofluorescence in CA1 in hippocampus. Magnification 20 X, Scale bar = 50 um. (n = 4/group) $^{*}P < 0.01$.

Effects of TQS on Iba-1 expression in DG in hippocampus

The effects of TQS on Iba-1 expression in DG region of the hippocampus are displayed in Figure 45. One way ANOVA indicated that TQS significantly decreased the expression of Iba-1 in LPS-induced neuroinflammatory pain ($F_{2,11} = 14.93$; $P < 0.001$). Multiple comparisons of means showed that LPS (1 mg/kg) significantly ($P < 0.001$) elevated the expression of Iba-1. Furthermore, TQS (4 mg/kg) significantly ($P < 0.05$) decreased Iba-1 expression in DG region of hippocampus.

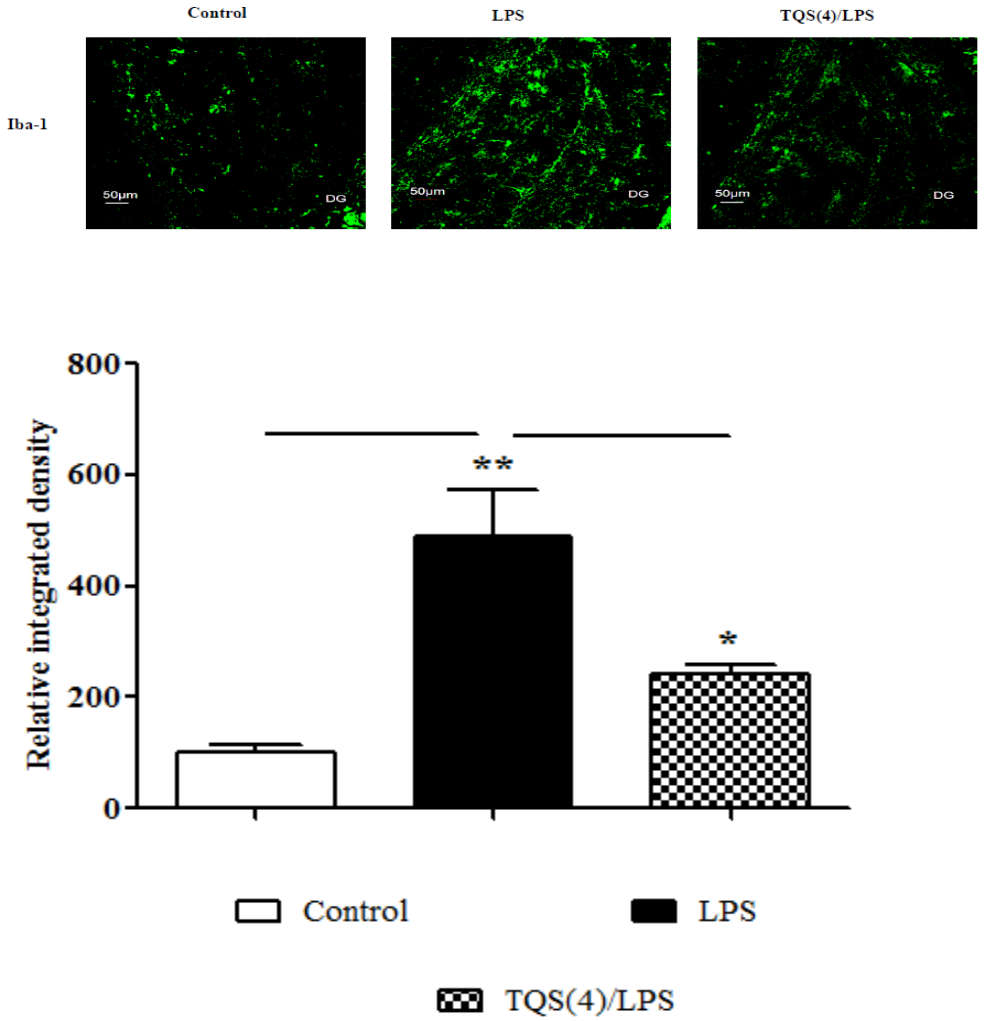

Figure 45: Lipopolysaccharide (LPS) significantly increased ionized calcium-binding adaptor molecule 1(Iba-1) expression compared to control in dentate gyrus (DG) region of hippocampus. Moreover, TQS significantly reduced the expression of Iba-1 compared to LPS treated group. Mice were administered TQS (4 mg/kg, i.p.) 0.5 h before LPS administration. Control mice received equal volume of vehicle. Whole brain was collected 6 h after LPS administration. The Iba-1 expression was quantified using immunofluorescence assay. Data are expressed as mean ± S.E.M. Representative images show the immunofluorescence in DG in hippocampus. Magnification 20 X, Scale bar = 50 um. (n = 4-5/group) $*P < 0.05$; $**P < 0.001$.

5.4. Discussion

The present study demonstrated that administration of TQS decreased the expression of BDNF in the hippocampus. TQS at same dose decreased neuroinflammatory pain-like symptoms as reported before (Abbas and Rahman, 2016). In support of this concept, co-administration of sub maximal dose of both TQS and ANA-12 produced antiallodynic and antihyperalgesic effects. This validated that TQS-mediated antiallodynic and antihyperalgesic effects of TQS are due to decreased BDNF expression in the hippocampus. Furthermore, TQS-induced decrease in the expression of BDNF mRNA was reversed by MLA confirming the involvement of α7 nAChR in TQS effects. TQS also decreased the immunoreactivity of Iba-1, a microglial activation marker, in CA1 and DG regions of hippocampus showing TQS decreases neuroinflammatory pain through reducing microglial activation in the hippocampus. Taken together, these data strongly indicate that reduced expression of microglial BDNF is critically involved in the mediation of TQS-induced antiallodynic and antihyperalgesic effects involving α7 nAChR in our mouse model for neuroinflammatory pain.

Our data are consistent with this view that microglial BDNF is a key mediator in LPS-induced neuroinflammatory pain model. For example, BDNF induces painful response in neuroinflammatory pain conditions (Groth and Aanonsen, 2002; Trang et al., 2011). A pervious study indicated that the expression of BDNF is significantly elevated in animal model of spinal nerve ligation (Li et al., 2006) associated with microglial activation (Coull et al., 2005; Cragg et al., 2010). Microinjection of BDNF into spinal cord causes increased pain behavior in animal models (Zhou et al., 2000). Furthermore,

intraplantar injection of BDNF also causes in increased pain like symptoms in animal models (Shu and Mendell, 1999).

The BDNF is proposed to enhance pain behavior due to increased activation of NKCC1 and reduced KKC2 expression (Price et al., 2005). Anti-BDNF antibodies have been shown to reduce pain behavior in animal models (Quintao et al., 2008). Moreover, administration of TrkB antibody also decreased the development of painful symptoms (Yajima et al., 2002). Additionally, sequestering BDNF using TrkB-Fc fusion protein also decreased the development of pain symptoms. Furthermore, Knockdown of BDNF using siRNA decreases the development of pain behavior (Coull et al., 2005). Therefore, eliminating BDNF reduces neuroinflammatory pain in animal models (Zhao et al., 2006). Our observations, of coincident effects of TQS on the pain behavior and the down-regulation of the BDNF pathway lend credence to the above findings and validate a link between enhanced BDNF expression and pain hypersensitivity in LPS-induced neuroinflammatory pain model in mice. It is noteworthy to mention that, LPS failed to increase BDNF expression in the brain stem (data not shown), validating the BDNF expression in specific brain region (i.e. hippocampus) in neuroinflammatory pain. Together, these findings indicate the possibility that BDNF might be required for the development of inflammatory pain in animal models.

Our findings showed that co-administration of TQS with ANA-12 had synergistic effects in decreasing neuroinflammatory pain-like symptoms suggesting the involvement of BDNF-mediated signaling in microglial cells during antiallodynic and antihyperalgesic effects. Moreover, the pretreatment of MLA, an α7 nAChR antagonist, reversed TQS-induced decrease in the expression of BDNF mRNA in the hippocampus validating the

involvement of α7 nAChRs. To the best of our knowledge, this is the first report which shows that occupancy of microglial α7 nAChR positive allosteric modulatory site in the hippocampus results in reduced BDNF expression in neuroinflammatory pain. We propose that microglial BDNF might need to be inhibited for the blockade of LPS-induced neuroinflammatory pain.

Our results showed that LPS enhanced BDNF expression in DG and CA1 regions, hippocampal regions involved in pain processing and perception, in LPS-induced painful conditions. TQS, however, resulted in marked reduction in the expression of BDNF in these both regions indicating that TQS decreases painful behavior via reduced BDNF expression in DG and CA1. However, further studies are needed involving direct microinjection of LPS and/or TQS in the hippocampus and to examine change in BDNF expression directly in the hippocampus.

The fact is that enhanced expression of BDNF in CA1 and DG regions of the hippocampus is associated with microglial activation can be inferred by increased expression of Iba-1, a microglial activation marker, in neuroinflammatory pain. We propose that BDNF positive cells are microglial cells that are highly implicated pain hypersensitivity. We would hypothesize that activated microglial cells in these both regions of the hippocampus hypersensitize the neuronal elements through downstream signaling mediating painful behavior. It is, therefore, possible that key effects of TQS on microglial α7 nAChR positive allosteric modulatory sites within CA1 and DG regions of the hippocampus are due to microglial cells, as LPS-induced increased immunoreactivity of Iba-1 was significantly attenuated by TQS. However, the exact mechanism through which microglial BDNF affects painful behavior by hypersensitizing neurons is not

clearly known. Previous studies have shown that NKCC1 and KCC2 might be involved. BDNF plays central role in the regulation of NKCC1 and KCC2 involving TrkB pathway (Rivera et al., 2002; Rivera et al., 2004). Both NKCC1 and KCC2 are predominately expressed on neurons and plays very important role in maintaining neuronal chloride homeostasis. Moreover, inflammation has been shown to increase NKCC1 activity and decreases KCC2 expression resulting in increased pain sensitivity due to GABA-mediated dis-inhibition. Therefore, further studies are warranted to examine the involvement of NKCC1 and KCC2 in LPS-induced neuroinflammatory pain in the hippocampus.

In conclusion, our results demonstrate that LPS-induced tactile allodynia and thermal hyperalgesia involve increased expression of microglial BDNF and associated pathway involving microglial α7 nAChR. Moreover, our study has show that TQS decreases neuroinflammatory pain due to reduced BDNF expression in the hippocampus. Overall, we propose that inhibition of microglial BDNF expression is a critical and novel therapeutic target for the clinical utility of neuroinflammatory pain.

Chapter 6: Effects of α7 nAChR PAM on NKCC1 and KCC2 expression and NKCC1 activation in the hippocampus

6.1. Introduction

As reported in chapter 5, the BDNF released from activated microglia is critically involved in the expression of NKCC1 and KCC2 and NKCC1 activation (Benarroch, 2010; Coull et al., 2005; De Koninck, 2007; Ellis and Bennett, 2013). In the CNS, both NKCC1 and KCC2 are major players in maintaining intracellular regulation of Cl^- (Kaila, 1994). The NKCC1 is involved for the movement of Cl^- into the cell (Haas, 1994; Plotkin et al., 1997) while KKC2 causes Cl^- movement out of cell thus maintaining neuronal homeostasis (Delpire and Mount, 2002; Payne et al., 1996; Plotkin et al., 1997). The activity of both of these cation-chloride co-transporters is involved in control and regulation of neuronal excitability. The function of NKCC1 is regulated by NKCC1 phosphorylation (the active form of NKCC1) and show a mechanism for increased activity of this transporter (Darman and Forbush, 2002).

Previous studies have indicated that NKCC1 and KCC2 play a very critical role in the generation and maintenance of neuroinflammatory painful conditions and their expression is altered in neuroinflammatory pain (Coull et al., 2003; Galan and Cervero, 2005; Nomura et al., 2006). For example the increased gene expression for NKCC1 results in painful responses while NKCC1 knock-out mice tolerate heat stimulus (Laird et al., 2004; Sung et al., 2000). Moreover, acute inflammatory pain was reported to be associated with increased activity of NKCC1 and reduced KKC2 expression (Galan and Cervero, 2005; Lee et al., 2014; Nomura et al., 2006).Taken together, these studies show that increased activity of NKCC1 and reduced KCC2 activity might be involved in pain

hypersensitivity and blocking NKCC1 expression and activation and increasing KCC2 expression might be a novel target for neuroinflammatory pain treatment.

Bumetanide (BU) is a loop diuretic used for the treatment of edema due to hepatic, renal disease, and congestive heart failure (Hughes and Lustman, 1990; Ward and Heel, 1984)**.** The Bu possesses 500-fold greater affinity for NKCC1 (Ki value of approximately 0.1 mM) than for KCC2 (Ki of approximately 25–50 mM). Therefore, Bu is relatively specific and potent inhibitor of NKCC1 and is an ideal tool to examine the role of NKKC1 in the hippocampus (Cramer et al., 2008; Kahle and Staley, 2008; Klein et al., 1999; Ko et al., 2014; Price et al., 2005). As described in chapter 5, TQS reduces neuroinflammatory pain by decreasing NF- κB activation and BDNF expression in the hippocampus. However, the effects of TQS on NKCC1 and KCC2 expression and NKCC1activation in the hippocampus are not known.

We hypothesized that TQS will decrease NKCC1 and KCC2 expression and NKCC1 activation in the hippocampus during antiallodynic and antihyperalgesic effects as reported earlier (Abbas and Rahman, 2016). Therefore, we have determined the effects of TQS on NKCC1 and KCC2 expression and NKCC1 activation in the hippocampus. In addition, we have examined the effects of TQS on phosphorylated cyclic adenosine monophosphate response element binding protein (p-CREB) expression in the hippocampus in mice.

6.2. Methods

Animals

Male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed under standard laboratory conditions as mentioned before (see chapter 2, section 2.2).

Drug treatment

The BU was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in normal saline containing 0.25% NaOH. The BU (10 or 30 mg/kg) was administered 1 h before the measurement of pain behavior (Cramer et al., 2008). The source, method of reconstitution, and time of administration for other drugs used in this study are mentioned previously (see chapter 2, section 2.2). Control animals were administered an equal volume of vehicle. The doses for BU were chosen based on previous report (Cramer et al., 2008).

Tactile allodynia

Tactile allodynia was measured as described before (see chapter 2, section 2.2).

Thermal hyperalgesia

Thermal hyperalgesia was quantified as mentioned earlier (see chapter 2, section 2.2).

RNA isolation and cDNA synthesis

Total RNA was isolated from hippocampal tissue as described previously (see chapter 3, section 3.2).

Quantitative real-time PCR

Quantitative real-time PCR was performed as mentioned previously (see chapter 3, section 3.2). Primers sequences were obtained from Integrated DNA Technologies (Coralville, Iowa, USA) and are given in Table 4.

Table 4: Prime primer sequence for GAPDH, NKCC1, and KCC2

Gene	**Primer sequence (5'-3')**
GAPDH	GTGGAGTCATACTGGAACATGTAG (forward)
	AATGGTGAAGGTCGGTGTG (reverse)
NKCC1	GGTATCATTAACATTGCCAGTGG (forward)
	CAGATCCTCAGTCAGCCATAC (reverse)
KCC2	AGCCTATGACGATGACCCA (forward)
	CCACCTCTGCTGTCTACATC (reverse)

Western blot analysis

Western blot analysis was conducted as previously described in detail (see chapter 2, section 2.2). Briefly, equal amounts of protein (50 μg) were loaded onto 10% gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis. Membranes were then blocked on a gyro-rocker with 5% nonfat dry milk in Tris-buffered saline/0.1% tween-20 for 50 min, and subsequently incubated overnight at 4°C with primary antibodies for p-CREB (1:500, rabbit polyclonal, Santa Cruz Biotech, USA), or β-tubulin (E7-S, 1:5,000, mouse monoclonal, University of Iowa, USA). After incubation, membranes were incubated with appropriate horseradish peroxide-conjugated secondary antibodies. Bound antibodies were detected with enhanced chemiluminescence prime reagent (Amersham,

Buckinghamshire, UK), and protein quantification was performed using densitometric analysis.

Immunofluorescence assay

Immunofluorescence assay was conducted as described previously (see chapter 3, section 3.2) using primary antibody for phospho-NKCC1 (EMD Millipore, Billerica, Massachusetts, USA).

Statistical analyses

For thermal hyperalgesia, Western blot analysis, immunofluorescence assay, and quantitative real-time PCR, data were analyzed by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. For quantitative real-time PCR for MLA and TQS/BU combination data for thermal hyperalgesia, Student's t-test was used. For tactile allodynia, Kruskil-Wallis test followed by Dunn's post hoc test for multiple comparisons or Mann-Whitney test for TQS/BU combination was applied using GraphPad Prism 5.0 (GraphPad Inc., San Diego, CA, USA). The difference between treatments was considered as significant at $P < 0.05$. All results are expressed as mean ± S.E.M.

6.3. Results

Effects of BU on 50% paw withdrawal in von Fey filament test in LPS-induced neuroinflammatory pain

The effects of BU on 50% paw withdrawal (tactile allodynia) during von Frey filament test are shown in Figure 46. Kruskal-wallis test showed that BU significantly affected 50% paw withdrawal threshold ($P < 0.0001$). Multiple comparisons of means

revealed that LPS (1 mg/kg) significantly ($P < 0.001$) decreased 50% paw withdrawal threshold compared to control showing the presence of pain hypersensitivity. Furthermore, BU (30 mg/kg) significantly ($P < 0.05$) enhanced 50% paw withdrawal threshold compared to LPS treatment group indicating antiallodynic effects of BU are due to the blockade of NKCC1.

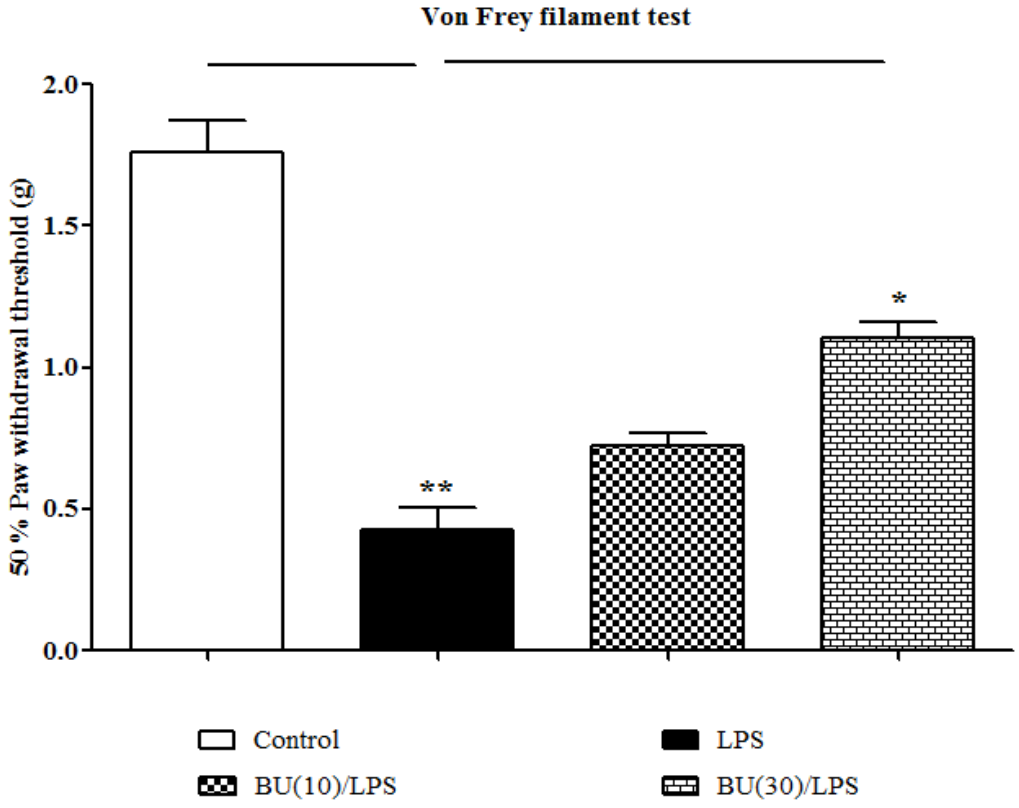

Figure 46: Lipopolysaccharide (LPS) significantly decreased 50% paw withdrawal threshold (tactile allodynia) compared to control. Moreover, bumetanide (BU) significantly increased 50% paw withdrawal threshold compared to LPS treated group. Mice received BU (10 or 30 mg/kg, i.p.) 5 h after LPS administration. Control animals received equal volume of vehicle. Paw withdrawal threshold was assessed using von Frey filaments six h after LPS administration. Data are expressed as mean $\pm$ S.E.M. (n = 5-8/group). $*P < 0.05$; $**P < 0.001$.

Effects of BU on latency time in hot plate test in LPS-induced neuroinflammatory pain

The effects of BU on latency time (thermal hyperalgesia) in hot plate test are shown in Figure 47. One way ANOVA showed that BU significantly altered latency time in hot plate test ($F_{3,19}$ = 8.794; $P < 0.001$). Multiple comparisons of means showed that LPS (1 mg/kg) significantly ($P < 0.001$) reduced hot plate latency as compared to control group indicating significant reduction in response to heat stimulus. Moreover, BU (30 mg/kg) significantly ($P < 0.05$) enhanced hot plate latency compared to LPS treatment group indicating antihyperalgesic effects of BU are due to the blockade of NKCC1.

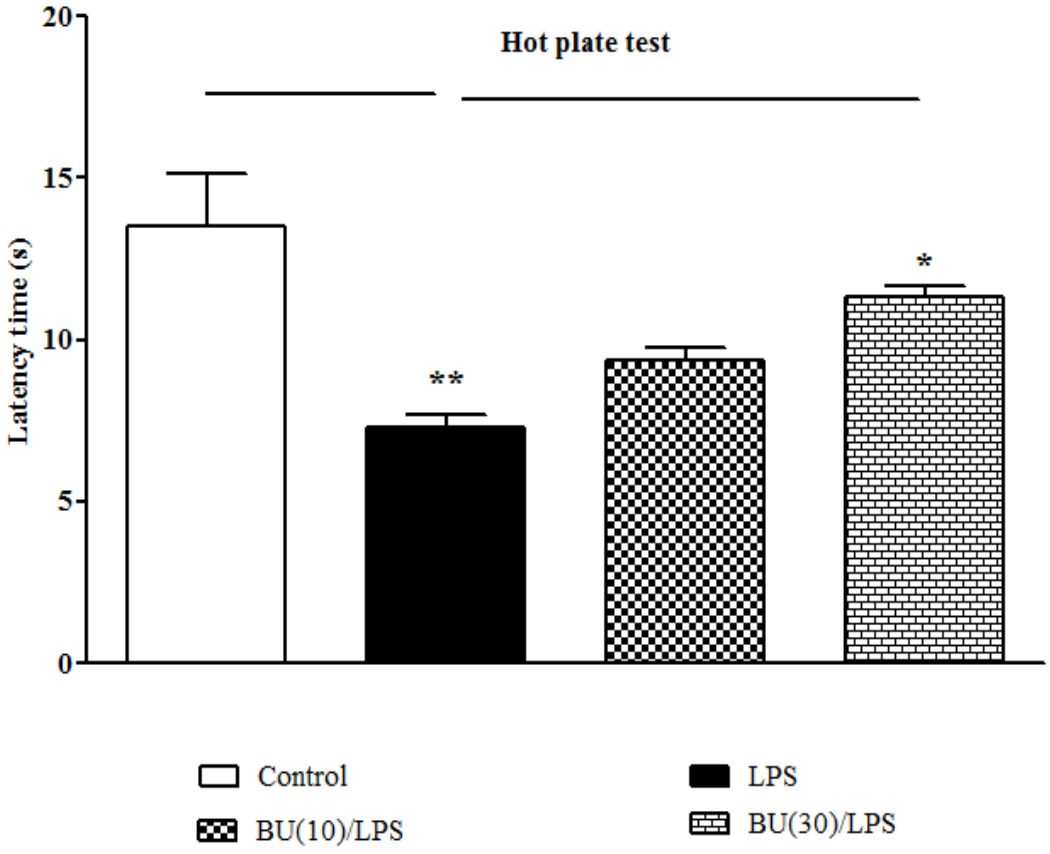

Figure 47: Lipopolysaccharide (LPS) significantly decreased latency time (thermal hyperalgesia) on the hot plate. Moreover, bumetanide (BU) significantly increased latency time on hot plate compared to LPS treated group. Mice received BU (10 or 30 mg/kg, i.p.) 5 h after LPS administration. Control animals received equal volume of vehicle. Latency on hot plate was measured six h after LPS administration. Data are expressed as mean $\pm$ S.E.M. (n = 5-6/group). $*P < 0.05$; $**P < 0.001$.

Effects of co-administration of TQS and BU on 50% paw withdrawal threshold in von Fey filament test in LPS-induced neuroinflammatory pain

The effects of co-administration of TQS and BU on 50% paw withdrawal threshold (tactile allodynia) are displayed in Figure 48. Mann-Whitney *test* showed that co-administration of TQS and BU significantly ($P < 0.01$) decreased 50% paw withdrawal threshold indicating reduction in the activation of NKCC1 during TQS-mediated antiallodynic response.

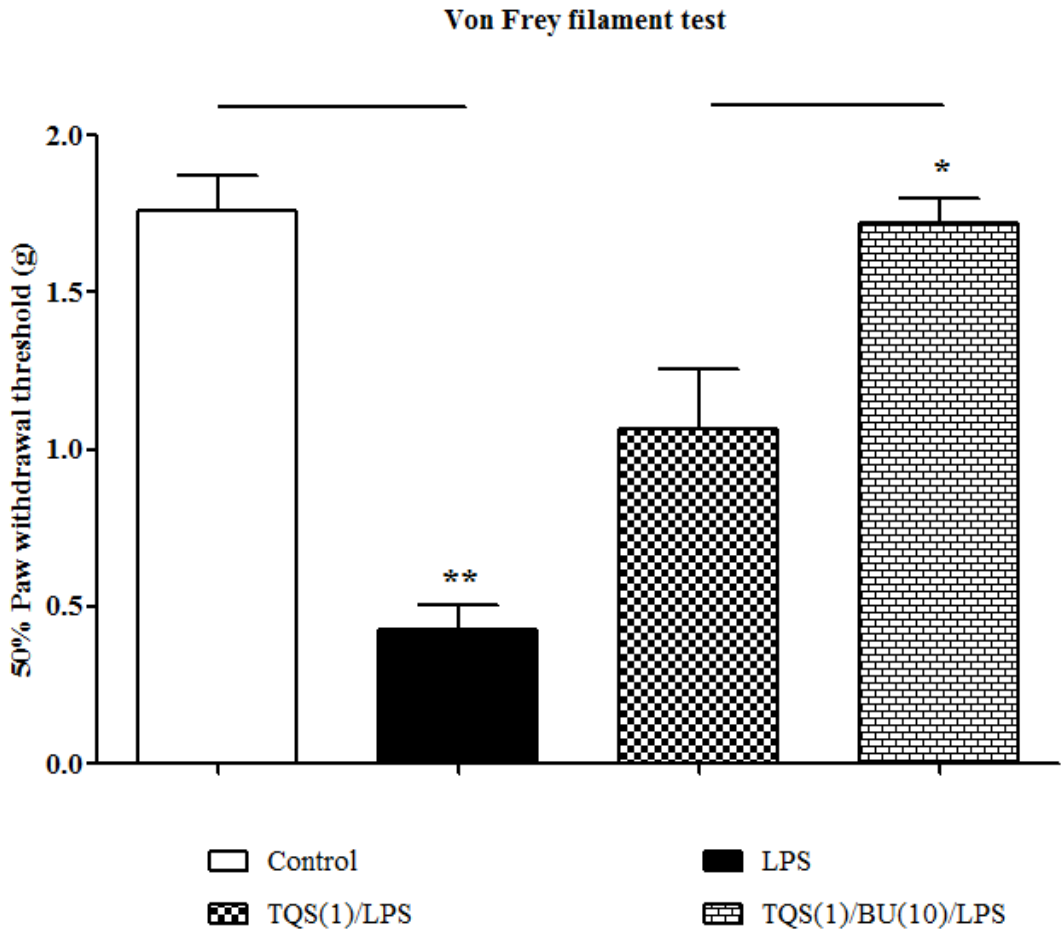

Figure 48: Lipopolysaccharide (LPS) significantly decreased 50% paw withdrawal threshold (tactile allodynia) compared to control. Furthermore, TQS in combination with bumetanide (BU) significantly increased 50% paw withdrawal threshold compared to TQS treated group. Mice received BU (10 mg/kg, i.p.) 5 h after LPS administration. TQS was given 30 minutes before LPS administration. Control animals received equal volume of vehicle. Paw withdrawal threshold was assessed using von Frey filaments six h after LPS administration. Data are expressed as mean $\pm$ S.E.M. (n = 6-8/group). *$P < 0.01$, **$P < 0.0001$.

Effects of co-administration of TQS and BU on latency time in hot plate test in LPS-induced neuroinflammatory pain

The effects of co-administration of TQS and BU on latency time (thermal hyperalgesia) are displayed in Figure 49. Student's t-test revealed that co-administration of TQS and BU significantly ($P < 0.01$) elevated latency time in hot plate test indicating antihyperalgesic effects of TQS are mediated through reduced NKCC1 activation.

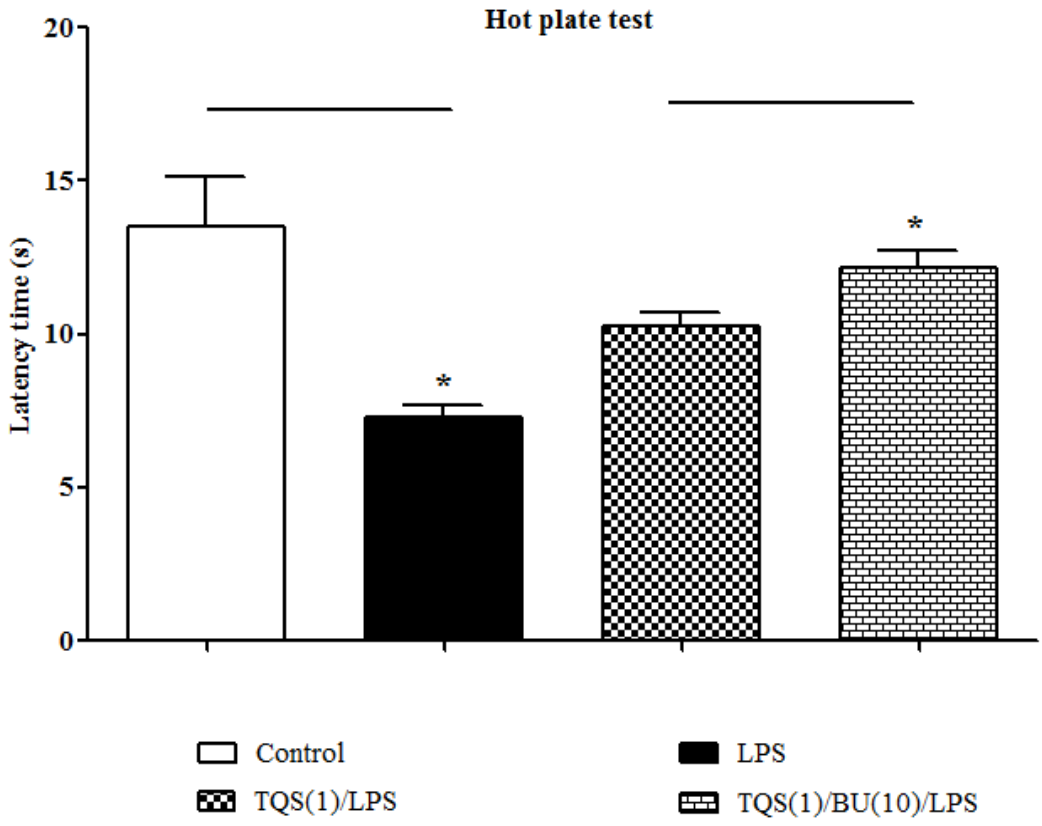

Figure 49: Lipopolysaccharide (LPS) significantly decreased latency time (thermal hyperalgesia) on the hot plate. Moreover, TQS in combination with bumetanide (BU) significantly increased latency time on hot plate compared to TQS treated group. Mice received BU (10 mg/kg, i.p.) 5 h after LPS administration. TQS was given 0.5 h before LPS administration. Control animals received equal volume of vehicle. Latency on hot plate was measured six h after LPS administration. Data are expressed as mean ± S.E.M. (n = 6/group). *$P < 0.01$.

Effects of TQS on NKCC1 mRNA expression in the hippocampus

The effects of TQS on NKCC1 mRNA expression are displayed in Figure 50. One way ANOVA showed that TQS did not significantly affect the expression of NKCC1 mRNA ($F_{2,9} = 1.556$; $P > 0.05$) in the hippocampus.

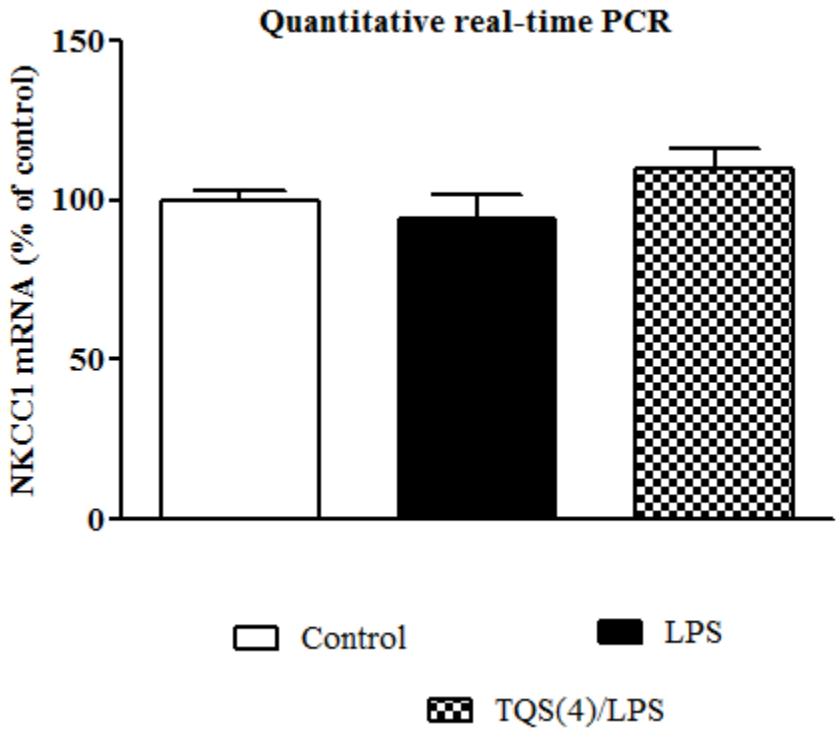

Figure 50: TQS did not significantly affect the expression of NKCC1in the hippocampus. Mice were administered TQS (4 mg/kg, i.p.) 0.5 h before LPS administration. Control animals received equal volume of vehicle. Hippocampi were collected 6 h after LPS administration. The NKCC1 mRNA expression was quantified using quantitative real-time polymerase chain reaction. (n = 4/group). Data are expressed as mean $\pm$ S.E.M.

Effects of TQS on KCC2 mRNA expression in the hippocampus

The effects of TQS on KCC2 mRNA expression are displayed in Figure 51. One way ANOVA showed that TQS significantly affected the expression of KCC2 mRNA ($F_{2,9}$ = 13.17; $P < 0.01$). Multiple comparisons of means showed that LPS (1 mg/kg) significantly ($P < 0.01$) reduced expression of KCC2 mRNA suggesting LPS-induced neuroinflammatory pain might be due to decreased the expression of KCC2. Furthermore, TQS (4 mg/kg) significantly ($P < 0.05$) increased the expression of KCC2 mRNA in LPS-induced neuroinflammatory pain supporting the hypothesis that TQS reduced neuroinflammatory pain-like symptoms by increasing KCC2 expression in the hippocampus.

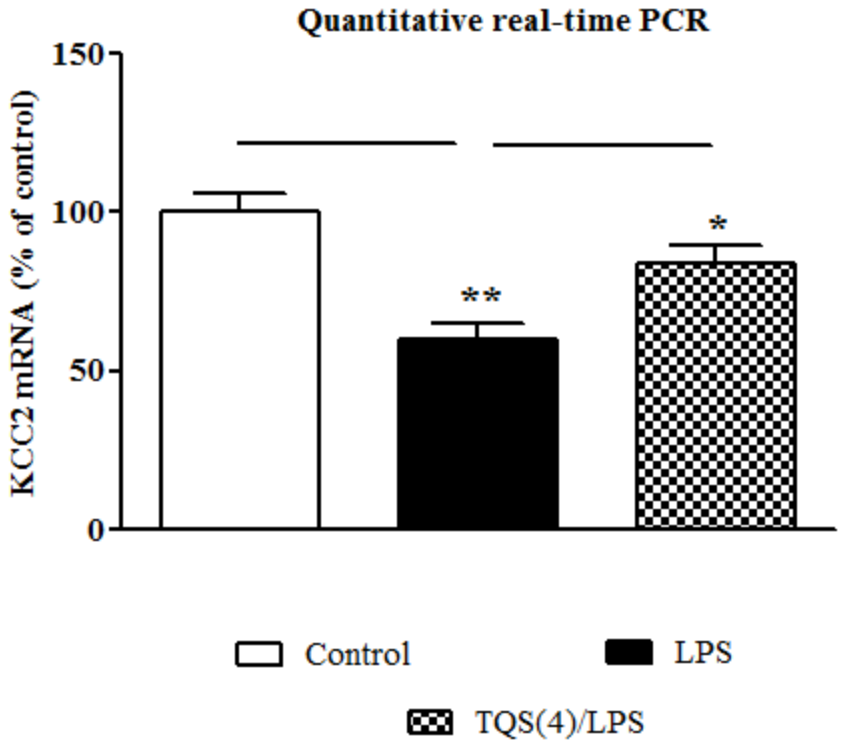

Figure 51: Lipopolysaccharide (LPS) significantly decreased K-Cl co-transporter 2 (KCC2) mRNA expression compared to control. Moreover, TQS significantly increased the expression of KCC2 mRNA compared to LPS treated group. Mice were administered TQS (4 mg/kg, i.p.) 0.5 h before LPS administration. Control animals received equal volume of vehicle. Hippocampi were collected 6 h after LPS administration. The KCC2 mRNA expression was quantified using quantitative real-time polymerase chain reaction. Data are expressed as mean $\pm$ S.E.M. (n = 4/group). *$P < 0.05$; **$P < 0.01$.

Effects of MLA on TQS-induced increase in KCC2 mRNA expression in the hippocampus

The effects of MLA on TQS-induced increase in KCC2 mRNA expression are displayed in Figure 52. Student's t-test indicated that MLA significantly ($P < 0.05$)

reversed TQS-induced increase in KCC2 mRNA expression. This confirmed that TQS-induced increase in KCC2 mRNA is mediated through α7 nAChR.

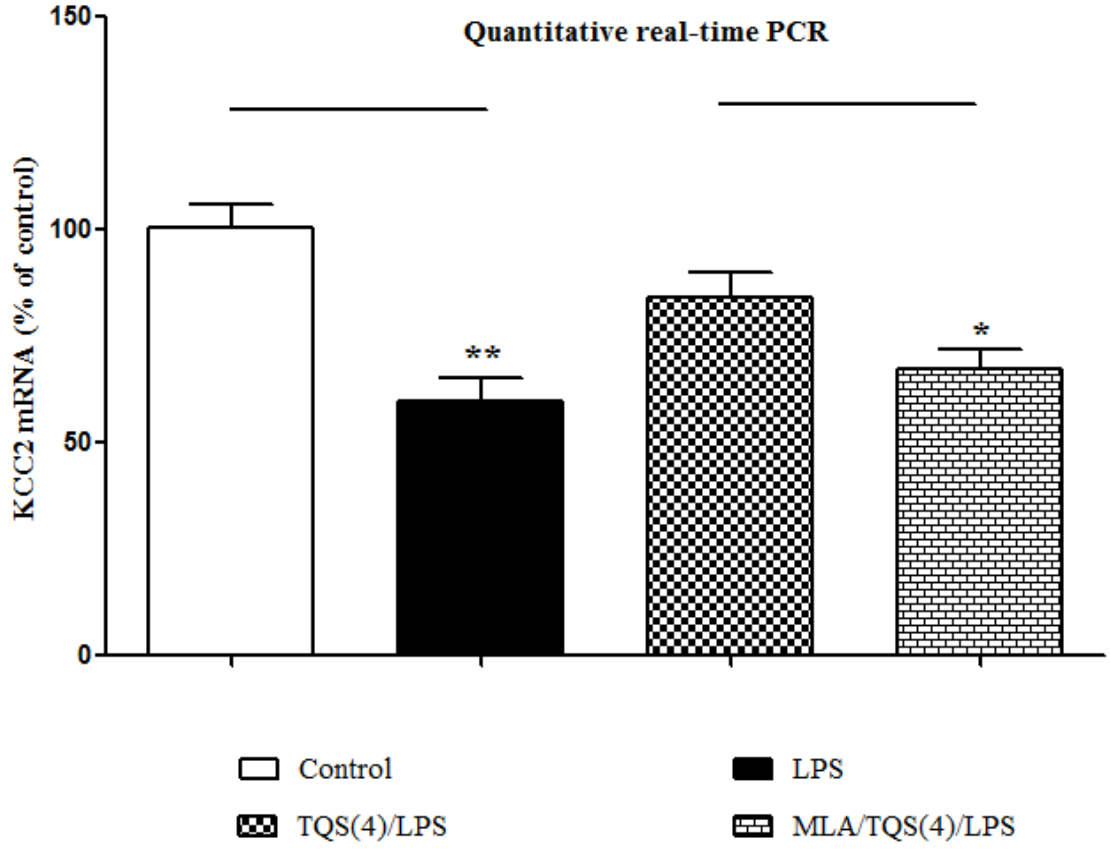

Figure 52: Methyllycaconitine (MLA) significantly decreased K-Cl co-transporter2 (KCC2) mRNA expression compared to TQS treatment group. Mice were administered TQS (4 mg /kg, i.p.) 0.5 h before LPS administration. The MLA (3 mg /kg, i.p.) was given 10 min before TQS injection. Control animals received equal volume of vehicle. Hippocampi were collected 6 h after LPS administration. The KCC2 mRNA expression was quantified using quantitative real-time polymerase chain reaction. Data are expressed as mean ± S.E.M. (n = 4/group). *$P < 0.05$; **$P < 0.01$.

Effects of TQS on p-NKCC1 expression in CA1 in hippocampus

The effects of TQS on p-NKCC1 expression in CA1 region of the hippocampus are displayed in Figure 53. One way ANOVA showed that TQS significantly altered p-NKCC1 expression in LPS-induced neuroinflammatory pain ($F_{2,11} = 13.91$; $P < 0.01$). Multiple comparisons of means showed that LPS (1 mg/kg) significantly ($P < 0.01$) elevated the expression of p-NKCC1. Furthermore, TQS (4 mg/kg) significantly ($P < 0.01$) decreased p-NKCC1 expression in CA1 region of hippocampus.

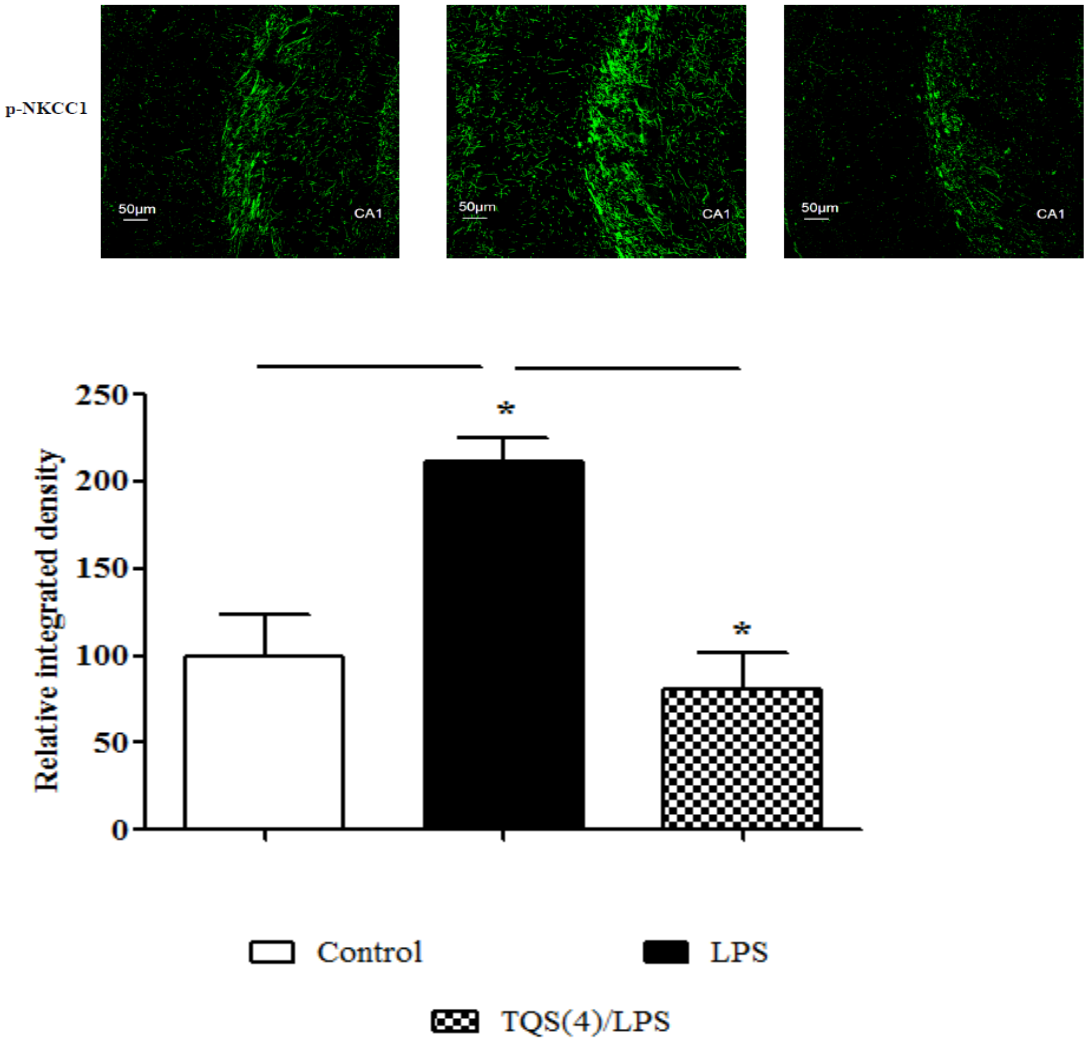

Figure 53: Lipopolysaccharide (LPS) significantly increased phospho-Na-K-Cl cotransporter1 (p-NKCC1) expression in CA1 region of hippocampus compared to control. Moreover, TQS significantly reduced the expression of p-NKCC1 compared to LPS treated group. Mice were administered TQS (4 mg/kg, i.p.) 0.5 h before LPS administration. Control mice received equal volume of vehicle. Whole brain was collected 6 h after LPS administration. The p-NKCC1 expression was quantified using immunofluorescence assay. Data are expressed as mean $\pm$ S.E.M. Representative images show the immunofluorescence in CA1 in hippocampus. Magnification 20 X, Scale bar = 50 um. (n = 4-5/group). $^{*}P < 0.01$.

Effects of TQS on p-NKCC1 expression in DG in hippocampus

The effects of TQS on p-NKCC1 expression in DG region of the hippocampus are displayed in Figure 54. One way ANOVA showed that TQS significantly altered p-NKCC1 expression in LPS-induced neuroinflammatory pain ($F_{2,9} = 16.84$; $P < 0.001$). Multiple comparisons of means showed that LPS (1 mg/kg) significantly ($P < 0.01$) elevated the expression of p-NKCC1. Furthermore, TQS (4 mg/kg) significantly ($P < 0.001$) decreased p-NKCC1 expression in DG region of hippocampus.

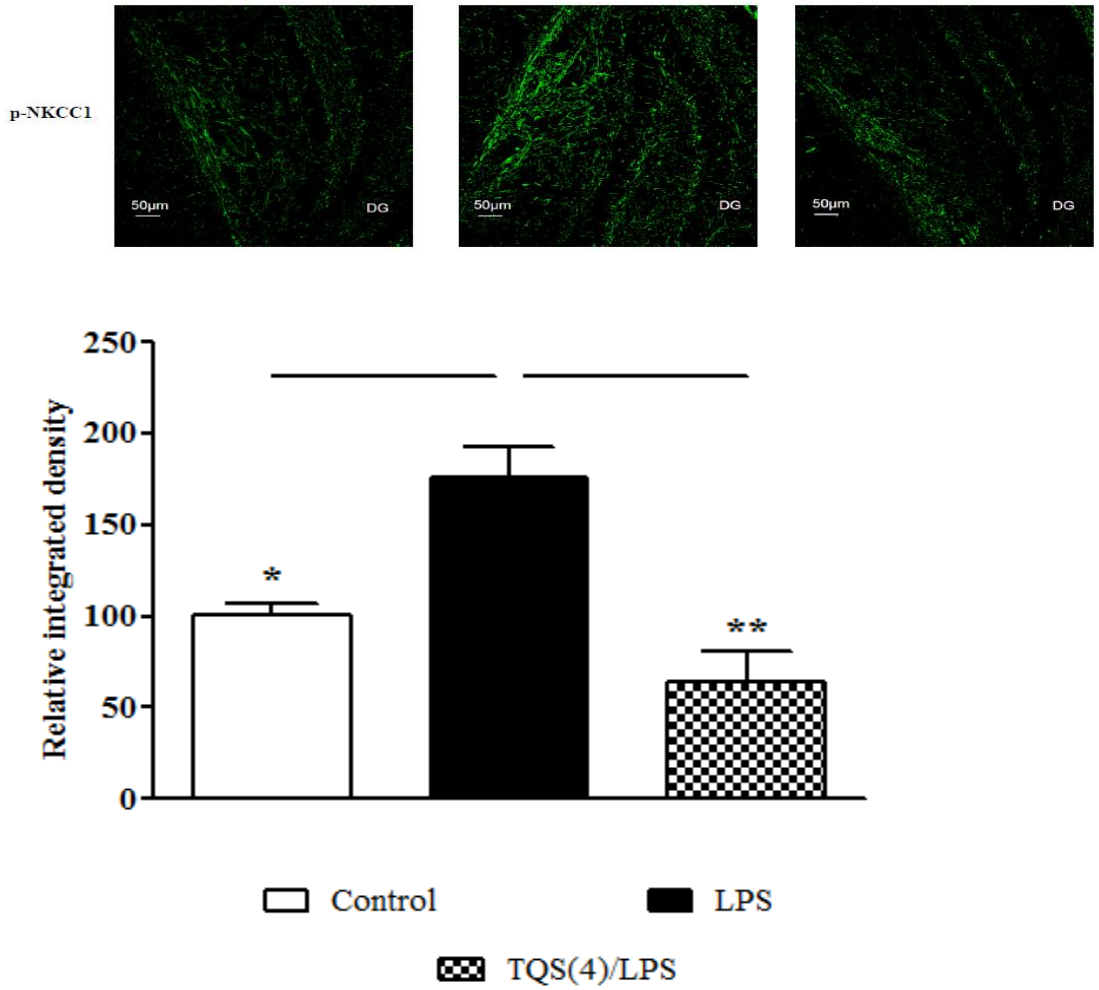

Figure 54: Lipopolysaccharide (LPS) significantly increased phospho-Na-K-Cl cotransporter1 (p-NKCC1) expression in dentate gyrus (DG) of hippocampus compared to control. Moreover, TQS significantly reduced the expression of p-NKCC1 compared to LPS treated group. Mice were administered TQS (4 mg/kg, i.p.) 0.5 h before LPS administration. Control mice received equal volume of vehicle. Whole brain was collected 6 h after LPS administration. The p-NKCC1 expression was quantified using immunofluorescence assay. Data are expressed as mean $\pm$ S.E.M. Representative images show the immunofluorescence in DG in hippocampus. Magnification 20 X, Scale bar = 50 um. (n = 4/group) $*P < 0.01$; $**P < 0.001$.

Effects of TQS on p-CREB expression in the hippocampus

The effects of TQS on p-CREB expression are displayed in Figure 55. One way ANOVA showed that TQS did not significantly affect the expression of P-CREB ($F_{3,12}$ = 0.1744; P = 0.9116) supporting the hypothesis that TQS did not affect neuronal α7 nAChR in the hippocampus.

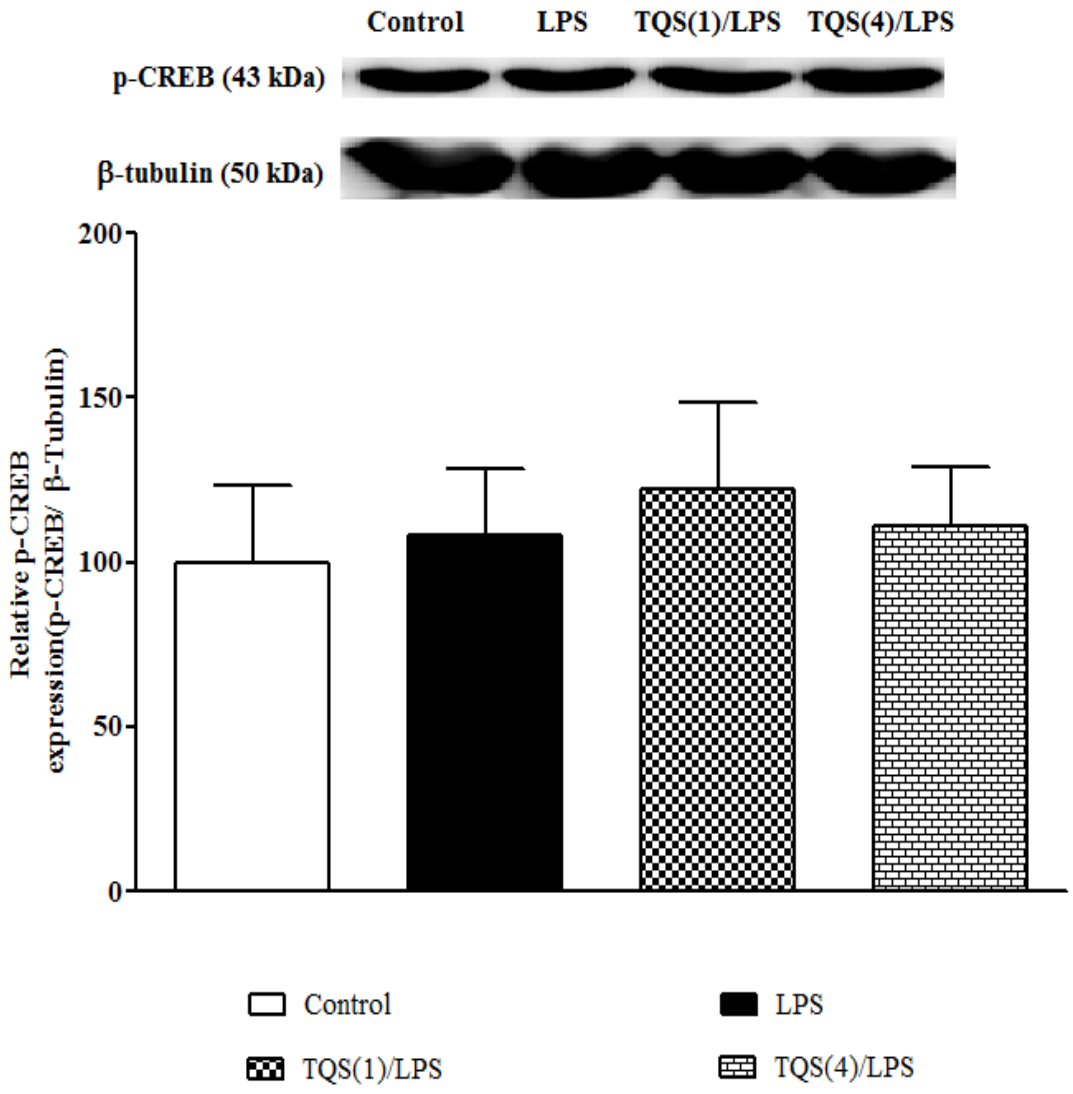

Figure 55: Lipopolysaccharide (LPS) did not significantly affect phosphorylated cyclic adenosine monophosphate response element binding protein (p-CREB) expression compared to control. Moreover, TQS did not reduce the expression of p-CREB compared to LPS treated group. Mice were administered TQS (4 mg/kg, i.p.) 0.5 h before LPS administration. Control animals received equal volume of vehicle. Hippocampi were collected 6 h after LPS administration. The p-CREB expression was quantified using quantitative real-time polymerase chain reaction. (n = 4/group). Data are expressed as mean ± S.E.M.

6.4. Discussion

The present study indicated that administration of TQS increased KCC2 expression and reduced NKCC1 activation in the hippocampus. TQS at same dose decreased neuroinflammatory pain-like symptoms as reported earlier (Abbas and Rahman, 2016). In support of this concept, co-administration of sub maximal dose of both TQS and BU produced antiallodynic and antihyperalgesic effects. This validates TQS-mediated antiallodynic and antihyperalgesic effects are due to decreased NKCC1 activation in the hippocampus. Furthermore, TQS-induced increase in KCC2 mRNA expression was reversed by MLA confirming the involvement of α7 nAChR in TQS effects. TQS also decreased the immunoreactivity of phospho-NKCC1, the active form of NKCC1, in CA1 and DG regions of hippocampus indicating TQS decreases neuroinflammatory pain through reducing NKCC1 activation in the hippocampus. Taken together, these data strongly suggest that increased expression of KCC2 and reduced activation of NKCC1 is critically involved in the mediation of TQS-induced antiallodynic

and antihyperalgesic effects involving α7 nAChR positive allosteric modulation in our mouse model for neuroinflammatory pain.

Our data are consistent with this view that neuronal NKCC1 and KCC2 are key elements in LPS-induced neuroinflammatory pain model. Dysregulation of NKCC1 and KCC2 were shown to be involved in the pathophysiology of neuroinflammatory pain. For example, acute inflammatory pain was reported to be due to increased expression of NKCC1 and reduced KCC2 expression in CNS (Galan and Cervero, 2005; Nomura et al., 2006). The NKCC1 knockout mice were shown to exhibit reduced neuroinflammatory pain-like symptoms (Sung et al., 2000). Our observations, of coincident effects of TQS on the pain behavior and decreased NKCC1 activation and increased KCC2 expression provide support to above findings. This validated a link between decreased NKCC1 activation and increased KKC2 expression, and reduced pain hypersensitivity in LPS-induced neuroinflammatory pain model in mice. Together, these findings suggest that NKCC1 and KCC2 might be required for inflammatory pain in animal models. For example, inflammation was shown to increase NKCC1 activation and reduce KCC2 activity resulting in increased painful response (Nomura et al., 2006; Payne et al., 2003; Price et al., 2005; Valencia-de Ita et al., 2006). Furthermore, phosphorylation of NKCC1 and decreased KCC2 expression result in increased pain behavior (Galan and Cervero, 2005; Nomura et al., 2006) . Thus, activated NKCC1, was found to induce neuroinflammatory pain-like symptoms in animal models (Lee et al., 2014).

Our findings showed that co-administration of TQS with BU had synergistic effects in decreasing neuroinflammatory pain-like symptoms which indicates involvement of NKCC1 in antiallodynic and antihyperalgesic effects. Moreover, the

pretreatment of MLA, an α7 nAChR antagonist, reversed TQS-induced increase in KCC2 mRNA expression in the hippocampus validating the involvement of α7 nAChRs. To the best of our knowledge, this is the first report which shows that α7 nAChR positive allosteric modulation in the hippocampus causes increased KCC2 expression and reduced NKCC1 activation in neuroinflammatory pain. We propose that NKCC1 activation need to be inhibited and KCC2 expression might be increased for the blockade of LPS-induced neuroinflammatory pain.

Our results indicated that LPS increased the phosphorylation of NKCC1 (activated NKCC1) in DG and CA1 regions, hippocampal regions involved in pain processing and perception, in LPS-induced painful conditions. TQS, however, resulted in marked decrease in NKCC1 activation in these both regions indicating that TQS reduces painful behavior via decreased activity in DG and CA1. However, further studies are warranted involving direct microinjection of LPS and/or TQS in the hippocampus.

As reported earlier α7 nAChRs are found both on neuronal and non-neuronal cells, including microglial cells (Pocock and Kettenmann, 2007; Taly et al., 2009). Signaling through microglial α7 nAChRs is different from those neuronal α7 nAChRs (Suzuki et al., 2006). For example, a previous study has shown that signaling through activated neuronal α7 nAChR involves increased expression of p-CREB (Dajas-Bailador and Wonnacott, 2004). However, microglial α7 nAChR-mediated signaling involves decreased expression of NF-κB (Egea et al., 2015; Loram et al., 2010). Our results showed there was no change in the expression of p-CREB in the hippocampus excluding the involvement of neuronal α7 nAChR. Furthermore, as reported in chapter 3, TQS pretreatment decreased the activation of NF-κB indicative of microglial α7 nAChR

involvement. Moreover, changes in microglial morphology, reduced expression of Iba-1 and CD11b mRNA after TQS administration in the hippocampus are suggestive of involvement of microglia in LPS-induced neuroinflammatory pain. We, therefore, propose that TQS-mediated antiallodynic and antihyperalgesic effects are due to microglial α7 nAChR activation and associated pathway.

In conclusion, our study determined the effects of reduced KCC2 expression and active NKCC1 in pain facilitation in the hippocampus in neuroinflammatory pain. Overall, we propose that inhibition of NKCC1 activation and enhancement of KCC2 expression are critical and novel therapeutic approach for the clinical utility of neuroinflammatory pain.

Chapter 7: General Discussion

The findings presented in this dissertation determined the effects of microglial α7 nAChR positive allosteric modulation-mediated signaling in the hippocampus in LPS-induced neuroinflammatory pain associated with microglial activation using widely established mouse models.

In chapter 2, we found that TQS significantly decreased tactile allodynia and thermal hyperalgesia in LPS-induced neuroinflammatory pain. Pretreatment with MLA reversed TQS-induced antiallodynic and antihyperalegesic effects validating the involvement of α7 nAChR in the mediation of TQS effects in reduced pain behavior. Our results are consistent with previous findings (Loram et al., 2010) and support the notion that activating α7 nAChR reduces pain hypersensitivity in our mouse models of neuroinflammatory pain. Furthermore, we demonstrated that TQS significantly reduced the expression of Iba-1 in the hippocampus indicating reduced microglial activation. Previous study also showed that decreasing microglial activation reduces neuroinflammatory pain involving reduced Iba-1 expression in spinal cord (Yoon et al., 2012). Microglial activation has been found to be associated with increased NF-κB activation (Loram et al., 2010). Our results are novel regarding the role of microglial cells in hippocampus in neuroinflammatory pain as hippocampus is a less studied supraspinal area with regard to pain perception and processing. Furthermore, hippocampus is connected to other brain areas that play critical role in modulating pain behavior. The microglial inhibitors such as propentofylline decreases pain hypersensitivity (Sweitzer et al., 2001). Conversely, fractalkine, the microglial activator, produces behavioral signs of neuroinflammatory pain, including allodynia, and hyperalgesia (Milligan et al., 2004).

TQS alone did not produce antiallodynic and antihyperalgesic effects, indicating TQS reduces tactile allodynia and thermal hyperalgesia asoociated with microglial activation. Overall, our results indicate the critical role of microglial α7 nAChR in LPS-induced neuroinflammatory pain and provide strong evidence that positive allosteric modulation is a novel approach to reduce neuroinflammatory pain associated with microglial activation in the hippocampus. Therefore, α7 nAChR PAM can be developed as potential drug candidate for neuroinflammatory pain.

In order to examine the effects of microglial α7 nAChR positive allosteric modulation-mediated signaling, we determined the effects of TQS on NF-κB activation as described in chapter 3. Our results showed that TQS reduced the expression of IκB, a downstream mediator which decreases as NF-κB becomes inactivated, indicating NF-κB inactivation. TQS also reduced microglial activation by decreasing the expression of CD11b mRNA, a microglial activation marker, in the hippocampus. Pretreatment with sub maximal dose of TQS and TEL reduced LPS-induced tactile allodynia and thermal hyperalgesia. In addition to reduced neuroinflammatory pain-like symptoms, co-administration of TQS with TEL decreased LPS-induced increase in NF-κB and microglial activation by decreasing IκB mRNA and CD11b mRNA expression. This validated our hypothesis that TQS reduces neuroinflammatory pain by decreasing NF-κB activation associated with microglial activation. Our results are in line with previous studies and indicate that increased neuroinflammatory pain-like symptoms are associated with NF-κB activation in the CNS (Loram et al., 2010). TQS prevented LPS-induced increased immunoreactivity of p-NF-κB p^{65} in CA1 and DG of the hippocampus indicating microglial α7 nAChRs play important role in reducing neuroinflammatory pain mediated by microglial NF-κB activation. The inactivation of NF-κB might be due to inhibition of IκB degradation associated with

increased intracellular Ca^{+}. This results in reduced translocatuon of active NF-κB within nucleus and decreases gene expression for pain mediators, including TNF, and BDNF. Previous studies have indicated that NF-κB is novel target for pain management. For example, systemic administration of NF-κB inhibitor decreases neuroinflammatory pain-like symptoms (Tegeder et al., 2004). Also, intrathecal administration of ammonium pyrrolidinedithiocarbamate and SN50, NF-κB inhibitors, modulate neuroinflammatory pain-like symptoms in animal models (Ledeboer et al., 2005). Taken together, our findings in chapter 3 indicate that reducing NF-κB within hippocampus is a novel strategy for reducing neuroinflammatory pain involving microglial α7 nAChR positive allosteric modulation. Due to potential ability to reduce NF-κB activation, the α7 nAChR PAM might be de novel therapeutic agent for neuroinflammatory pain.

As indicated earlier, NF-κB is critically involved in the expression of TNF, therefore, in chapter 4, we examined the effects of TQS on TNF expression in the hippocampus. Our findings indicated that TQS significantly reduced TNF expression in the hippocampus. TQS (1 mg/kg) did not affect TNF mRNA expression but decreased TNF level in the hippocampus in LPS-induced neuroinflammatory pain. This could be due to involvement of other downstream signaling pathway involved in post transcriptional modification of TNF that needs to be explored. Furthermore, TQS also increased the level of NE in the hippocampus. Our results support that a relationship exists between TNF and NE level in the hippocampus and showed that decreasing TNF resulted in increased NE in the hippocampus. Previous studies also support the same concept involving inverse relationship between TNF and NE in the hippocampus (Covey et al., 2000; Ignatowski et al., 1999). According to this hypothesis, increasing TNF in the hippocampus results in the alteration of α2 adrenergic receptor coupling to Gi/Gs-

proteins (Sud et al., 2008). For example increased TNF increases Gi-protein coupling with α2 adrenergic receptor compared to Gs-protein. This results in reduced NE release in the hippocampus and increased pain sensitivity. However, decreasing TNF increases Gs-proteins coupling with α2 adrenergic receptor compared to Gi-proteins thus facilitating NE release in the hippocampus as seen as with TQS pretreatment after LPS administration. The MLA reversed TQS-induced decrease in TNF mRNA indicating the involvement of α7 nAChR in the expression of TNF. Pretreatment of IDA reversed TQS-induced antiallodynic and antihyperalgesic effects. The IDA also reduced TQS-mediated increase in NE level. This validated the involvement of α2 adrenergic recptor in the mediation TQS effects on tactile allodynia and thermal hyperalgesia and NE release. Furthermore, TQS reduced the immunoreactivity of TNF in DG and CA1 regions of the hippocampus. The findings from chapter 3 indicate that TNF and NE in the hippocampus are potential drug targets and decreasing TNF and increasing NE might be novel approach for the treatment of neuroinflammatory pain involving α7 nAChR positive allosteric modulation. Due to reduction in TNF expression and increased NE level, α7 nAChR PAM might be potential drug candidate for neuroinflammatory pain.

We determined the effects of TQS on BDNF expression in the hippocampus as shown in chapter 5. Our results indicated that LPS significantly increased the expression of microglial BDNF indicating painful behavior is directly associated with enhanced BDNF level in the hippocampus. In line with our results, previous study showed that increased expression of microglial BDNF plays a critical in the mediation of Painful behavior (Coull et al., 2005). Our results showed that acute treatment with TQS decreased the expression of BDNF in the hippocampus. The TQS (1 mg/kg) reduced

BDNF mRNA expression while it did not decrease BDNF indicating TQS (1 mg/kg) might not be bio-available enough to affect post transcriptional modification of BDNF in the hippocampus. The administration of sub maximal dose of TQS with ANA-12 decreased tactile allodynia and thermal hyperalgesia. These results, therefore, indicate that TQS reduces neuroinflammatory pain by decreasing BDNF expression in the hippocampus. Moreover, TQS reduced the expression of BDNF in CA1 and DG regions of the hippocampus. Previous studies showed that BDNF is critically involved in the expression of NKCC1 and KCC2 in neurons. The up regulation and increased activation of NKCC1 while down regulation of KCC2 in neurons cause neuroinflammatory pain development involving BDNF-TrkB pathway. These results, therefore, indicate that reduced expression of BDNF and BDNF-mediated signaling is critically involved in antiallodynic and antihyperalgesic effects of TQS. Since TQS decreases microglial BDNF expression, therefore, α7 nAChR PAM might be novel therapeutic agent for neuroinflammatory pain.

As reported in earlier, BDNF is involved in the expression of NKCC1 and KCC2 in neuroinflammatory pain, therefore, in chapter 6, we determined the effects of TQS on the regulation of NKCC1 and KCC2. Our findings showed that TQS reduced NKCC1 activation and increased KCC2 expression resulting in reduced neuroinflammatory pain-like symptoms. Moreover, the sub maximal dose of both TQS and BU reduced LPS-induced tactile allodynia and thermal hyperalgesia. This validated the involvement of NKCC1 in the mediation antiallodynic and antihyperalgesic effects of TQS. Our findings in this chapter indicate that TQS reduces neuroinflammatory pain by decreasing BDNF-induced neuronal hypersensitization involving BDNF-TrkB pathway. Previous studies

have reported that the activation of NKCC1 and decreased expression of KCC2 causes increased intracellular movement of Cl^{-1} within neurons. So, intracellular concentration of Cl^{-} builds up in the neurons. The binding of GABA at its receptor results in outward movement of Cl^{-1}. This results in depolarization and increased pain sensitivity as seen after LPS administration. However, further studies are warranted involving the measurement of Cl^{-1} within neurons. Our findings indicate that reduced expression of KCC2 and increased activation of NKCC1 play a critical role in pain hypersensitivity in neuroinflammation. Therefore, α7 nAChR PAM might be potential drug candidate for neuroinflammatory pain.

Overall, our findings revealed TQS decreased microglial TNF and BDNF expression and associated signaling in neuroinflammatory pain involving α7 nAChR and microglial activation in the hippocampus in mice. Therefore, TQS triggers microglial α7 nAChR positive allosteric modulation-mediated signaling pathway resulting in reduced painful symptoms in LPS-induced neuroinflammatory pain in mice as depicted in our proposed mechanism in Figure 56. Therefore, targeting α7 nAChR positive allosteric modulation on the microglia in the hippocampus is a novel strategy for the treatment of neuroinflammatory pain.

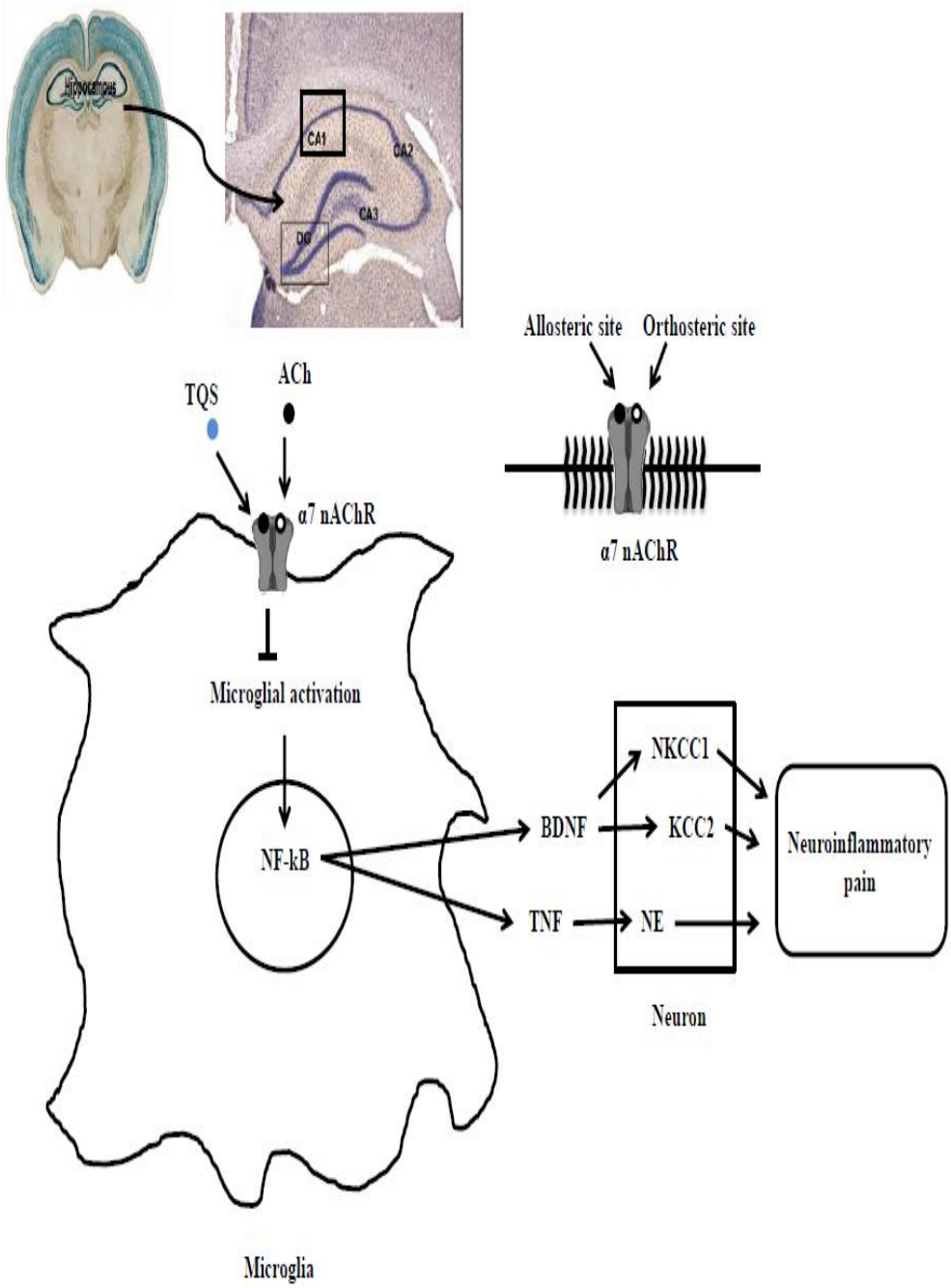
Hippocampus
CA1
CA2
CA3
DG
Allosteric site
Orthosteric site
TQS
ACh
α7 nAChR
α7 nAChR
Microglial activation
NF-kB
BDNF
TNF
NKCC1
KCC2
NE
Neuroinflammatory pain
Neuron
Microglia

Figure 56: Schematic for proposed signaling pathways involved in α7 nicotinic acetylcholine receptor (nAChR) positive allosteric modulation-mediated antiallodynic and antihyperalgesic effects in neuroinflammatory pain. The 3a,4,5,9b-tetrahydro-4-(1-naphthalenyl)-3*H*-cyclopentan[*c*]quinoline-8-sulfonamide (TQS) binds at allosteric site of α7 nAChR and acetylcholine (ACh) binds at orthosteric site on microglia in dentate gyrus and CA1 regions of the hippocampus. This causes decrease in nuclear factor-kappa-B (NF-κB), a transcription factor, activation through reduced microglial activation. This results in decrease in the induction and release of proinflammatory cytokines-tumor necrosis factor (TNF) and brain-derived neurotrophic factor (BDNF). Decreased TNF in the hippocampus disturbs the balance between Gαi-protein and Gαs protein and favors one functional form (coupled to Gαs) over the other (coupled to Gαi) at presynaptic α2-adrenergic autoreceptor. This increases norepinephrine (NE) release from noradrenergic neurons and decreases pain sensation. Moreover, decreased BDNF also reduces neuronal sodium-potassium-chloride co-transporter 1 (NKCC1) activation. Reduced BDNF also increases the expression of potassium-chloride co-transporter 2 (KCC2). This inhibits action potential generation and decreases pain sensitivity in neuroinflammatory pain. Modified and adopted from Covey et al. (2000) Brain Res 859**:**113-122; Gomes et al. (2013) J Neuroinflammation 10**:**16; Kahle et al. (2008) Nat Clin Pract Neurol 4**:**490-503; Loram et al. (2010) Brain Behav Immun 24**:**959-967; Miwa et al. (1997) J Neurosci Res 50**:**1023-1029; Price et al. (2005) Curr Top Med Chem 5**:**547-555 and Sud et al. (2008) Eur J Pharmacol 588**:**217-231.

Summary and conclusions

The main findings of this dissertation are given below:

i. Microglial α7 nAChR-mediated effects play a critical role in the reduction of neuroinflammatory pain involving reduced microglial activation in the hippocampus (chapter 2).

ii. Microglial α7 nAChR-mediated signaling decreases the activation of NF-κB in neuroinflammatory pain in the hippocampus (chapter 3).

iii. Microglial α7 nAChR-mediated signaling reduces the expression of TNF and increases NE level in neuroinflammatory pain in the hippocampus (chapter 4).

iv. Microglial α7 nAChR-mediated signaling modulates the expression of BDNF in neuroinflammatory pain in the hippocampus (chapter 5).

v. Microglial α7 nAChR-mediated signaling modulates the activation of NKCC1 and increases the expression KCC2 in hippocampal neurons in neuroinflammatory pain (chapter 6).

In conclusion, the current dissertation provided a novel discovery that microglial α7 nAChR positive allosteric modulation-mediated signaling regulates behavioral, cellular, biochemical, and molecular mechanisms underlying neuroinflammatory pain in the hippocampus in mice.

Future directions

The findings provided in this dissertation provide strong evidence for the critical role of microglial α7 nAChR positive allosteric modulation and associated signaling in neuroinflammatory pain. This gives rise to other future directions that include

i. We have examined the role of microglial cells and associated mechanisms in neuroinflammatory pain in the hippocampus in mice. Moreover, astrocytes play critical in the maintenance of neuroinflammatory pain, therefore futures studies are required to investigate the role of astrocytes in the hippocampus in neuroinflammatory pain.

ii. Our results have shown that proinflammatory cytokine, TNF plays a critical role in the development of neuroinflammatory pain. Previous studies indicate that other proinflammatory cytokines, including IL-1β, and IL6 are also involved in neuroinflammatory pain. Therefore, additional studies are needed to investigate the effects of IL-1β, and IL6 in the hippocampus in neuroinflammatory pain.

iii. We focused the involvement of hippocampus in LPS-induced neuroinflammatory pain. Additional studies are warranted to examine the role of other limbic regions including amagdyla, and anterior cingulated cortex in neuroinflammatory pain. Furthermore, the role of prefrontal cortex should be determined in neuroinflammatory pain.

iv. We have examined the effects of TQS in the hippocampus in neuroinflammatory pain in regular mice. Future studies involving the use of microglial α7 nAChR transgenic and knockout mice are required in order to further confirm the involvement of microglial α7 nAChR in neuroinflammatory pain.

REFERENCES

Aamodt, S., 2007. Focus on glia and disease. Nat Neurosci 10, 1349-1349.

Abbas, M., Rahman, S., 2016. Effects of alpha-7 nicotinic acetylcholine receptor positive allosteric modulator on lipopolysaccharide-induced neuroinflammatory pain in mice. Eur J Pharmacol 783, 85-91.

Abdrakhmanova, G. R., Blough, B. E., Nesloney, C., Navarro, H. A., Damaj, M. I., Carroll, F. I., 2010. In vitro and in vivo characterization of a novel negative allosteric modulator of neuronal nAChRs. Neuropharmacology 59, 511-517.

Al-Amin, H., Sarkis, R., Atweh, S., Jabbur, S., Saade, N., 2011. Chronic dizocilpine or apomorphine and development of neuropathy in two animal models II: effects on brain cytokines and neurotrophins. Exp Neurol 228, 30-40.

Alfonso Romero-Sandoval, E., Sweitzer, S., 2015. Nonneuronal central mechanisms of pain: glia and immune response. Prog Mol Biol Transl Sci 131, 325-358.

Ali, T., Badshah, H., Kim, T. H., Kim, M. O., 2015. Melatonin attenuates D-galactose-induced memory impairment, neuroinflammation and neurodegeneration via RAGE/NF-K B/JNK signaling pathway in aging mouse model. J Pineal Res 58, 71-85.

Alsharari, S. D., Freitas, K., Damaj, M. I., 2013. Functional role of alpha7 nicotinic receptor in chronic neuropathic and inflammatory pain: studies in transgenic mice. Biochem Pharmacol 86, 1201-1207.

Andurkar, S. V., Gendler, L., Gulati, A., 2012. Tramadol antinociception is potentiated by clonidine through alpha(2)-adrenergic and I(2)-imidazoline but not by endothelin ET(A) receptors in mice. Eur J Pharmacol 683, 109-115.

Apkarian, A. V., Sosa, Y., Sonty, S., Levy, R. M., Harden, R. N., Parrish, T. B., Gitelman, D. R., 2004. Chronic back pain is associated with decreased prefrontal and thalamic gray matter density. J Neurosci 24, 10410-10415.

Arias, H. R., 2010. Positive and negative modulation of nicotinic receptors. Adv Protein Chem Struct Biol 80, 153-203.

Atzori, M., Garcia-Oscos, F., Arias, H. R., 2016. α7 Nicotinic acetylcholine receptor-mediated anti-inflammatory actions modulate brain functions. Curr Signal Transduct Ther 3, e1303.

Austin, P. J., Moalem-Taylor, G., 2010. The neuro-immune balance in neuropathic pain: involvement of inflammatory immune cells, immune-like glial cells and cytokines. J Neuroimmunol 229, 26-50.

Bagdas, D., Sonat, F. A., Hamurtekin, E., Sonal, S., Gurun, M. S., 2011. The antihyperalgesic effect of cytidine-5'-diphosphate-choline in neuropathic and inflammatory pain models. Behav Pharmacol 22, 589-598.

Bagdas, D., Wilkerson, J. L., Kulkarni, A., Toma, W., AlSharari, S., Gul, Z., Lichtman, A. H., Papke, R. L., Thakur, G. A., Imad Damaj, M., 2016. The alpha7 nicotinic receptor dual allosteric agonist and positive allosteric modulator GAT107 reverses nociception in mouse models of inflammatory and neuropathic pain. Br J Pharmacol, 2506-2520.

Balaji, S. P., Ramanathan, M., 2014. Telmisartan Protects the Lipopolysaccharide Intoxicated RAW 264.7 Cell line by Deactivating NFκB Mediated Inflammatory Mechanism. J Pharm Sci Res 6, 68-72.

Baliki, M. N., Chialvo, D. R., Geha, P. Y., Levy, R. M., Harden, R. N., Parrish, T. B., Apkarian, A. V., 2006. Chronic pain and the emotional brain: specific brain activity

associated with spontaneous fluctuations of intensity of chronic back pain. J Neurosci 26, 12165-12173.

Baliki, M. N., Geha, P. Y., Apkarian, A. V., Chialvo, D. R., 2008. Beyond feeling: chronic pain hurts the brain, disrupting the default-mode network dynamics. J Neurosci 28, 1398-1403.

Bannon, A. W., Decker, M. W., Holladay, M. W., Curzon, P., Donnelly-Roberts, D., Puttfarcken, P. S., Bitner, R. S., Diaz, A., Dickenson, A. H., Porsolt, R. D., Williams, M., Arneric, S. P., 1998. Broad-spectrum, non-opioid analgesic activity by selective modulation of neuronal nicotinic acetylcholine receptors. Science 279, 77-81.

Bastos, L. F., Merlo, L. A., Rocha, L. T., Coelho, M. M., 2007. Characterization of the antinociceptive and anti-inflammatory activities of doxycycline and minocycline in different experimental models. Eur J Pharmacol 576, 171-179.

Beattie, E. C., Stellwagen, D., Morishita, W., Bresnahan, J. C., Ha, B. K., Von Zastrow, M., Beattie, M. S., Malenka, R. C., 2002. Control of synaptic strength by glial TNFalpha. Science 295, 2282-2285.

Beckmann, J., Lips, K. S., 2013. The non-neuronal cholinergic system in health and disease. Pharmacology 92, 286-302.

Benarroch, E. E., 2010. Central neuron-glia interactions and neuropathic pain: overview of recent concepts and clinical implications. Neurology 75, 273-278.

Bernardo, A., Minghetti, L., 2006. PPAR-gamma agonists as regulators of microglial activation and brain inflammation. Curr Pharm Des 12, 93-109.

Bertrand, D., Gopalakrishnan, M., 2007. Allosteric modulation of nicotinic acetylcholine receptors. Bioch Pharmacol 74, 1155-1163.

Bethea, J. R., Castro, M., Keane, R. W., Lee, T. T., Dietrich, W. D., Yezierski, R. P., 1998. Traumatic spinal cord injury induces nuclear factor-kappaB activation. J Neurosci 18, 3251-3260.

Bettoni, I., Comelli, F., Rossini, C., Granucci, F., Giagnoni, G., Peri, F., Costa, B., 2008. Glial TLR4 receptor as new target to treat neuropathic pain: efficacy of a new receptor antagonist in a model of peripheral nerve injury in mice. Glia 56, 1312-1319.

Borsook, D., 2012. Neurological diseases and pain. Brain 135, 320-344.

Bruchfeld, A., Goldstein, R. S., Chavan, S., Patel, N. B., Rosas-Ballina, M., Kohn, N., Qureshi, A. R., Tracey, K. J., 2010. Whole blood cytokine attenuation by cholinergic agonists ex vivo and relationship to vagus nerve activity in rheumatoid arthritis. J Intern Med 268, 94-101.

Callahan, P. M., Hutchings, E. J., Kille, N. J., Chapman, J. M., Terry, A. V., Jr., 2013. Positive allosteric modulator of alpha7 nicotinic-acetylcholine receptors, PNU-120596 augments the effects of donepezil on learning and memory in aged rodents and non-human primates. Neuropharmacology 67, 201-212.

Campbell, I. K., Gerondakis, S., O'Donnell, K., Wicks, I. P., 2000. Distinct roles for the NF-kappaB1 (p50) and c-Rel transcription factors in inflammatory arthritis. J Clin Invest 105, 1799-1806.

Cao, H., Zhang, Y. Q., 2008. Spinal glial activation contributes to pathological pain states. Neurosci Biobehav Rev 32, 972-983.

Carnevale, D., De Simone, R., Minghetti, L., 2007. Microglia-neuron interaction in inflammatory and degenerative diseases: role of cholinergic and noradrenergic systems. CNS Neurol Disord Drug Targets 6, 388-397.

Carter, G. T., Galer, B. S., 2001. Advances in the management of neuropathic pain. Phys Med Rehabil Clin N Am 12, 447-459.

Cazorla, M., Prémont, J., Mann, A., Girard, N., Kellendonk, C., Rognan, D., 2011. Identification of a low–molecular weight TrkB antagonist with anxiolytic and antidepressant activity in mice. J Clin Invest 121, 1846-1857.

Champtiaux, N., Changeux, J. P., 2002. Knock-out and knock-in mice to investigate the role of nicotinic receptors in the central nervous system. Curr Drug Targets CNS Neurol Disord 1, 319-330.

Changeux, J.-P., Edelstein, S. J., 2005. Allosteric mechanisms of signal transduction. Science 308, 1424-1428.

Chaplan, S. R., Bach, F. W., Pogrel, J. W., Chung, J. M., Yaksh, T. L., 1994. Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 53, 55-63.

Chatzidaki, A., D'Oyley, J. M., Gill-Thind, J. K., Sheppard, T. D., Millar, N. S., 2015. The influence of allosteric modulators and transmembrane mutations on desensitisation and activation of α7 nicotinic acetylcholine receptors. Neuropharmacology 97, 75-85.

Chen, Z., Jalabi, W., Shpargel, K. B., Farabaugh, K. T., Dutta, R., Yin, X., Kidd, G. J., Bergmann, C. C., Stohlman, S. A., Trapp, B. D., 2012. Lipopolysaccharide-induced microglial activation and neuroprotection against experimental brain injury is independent of hematogenous TLR4. J Neurosci 32, 11706-11715.

Cho, I. H., Chung, Y. M., Park, C. K., Park, S. H., Lee, H., Kim, D., Piao, Z. G., Choi, S. Y., Lee, S. J., Park, K., Kim, J. S., Jung, S. J., Oh, S. B., 2006. Systemic administration of minocycline inhibits formalin-induced inflammatory pain in rat. Brain Res 1072, 208-214.

Chou, C. W., Wong, G. T., Lim, G., McCabe, M. F., Wang, S., Irwin, M. G., Mao, J., 2011. Peripheral nerve injury alters the expression of NF-kappaB in the rat's hippocampus. Brain Res 1378, 66-71.

Christie, K. J., Turnley, A. M., 2012. Regulation of endogenous neural stem/progenitor cells for neural repair-factors that promote neurogenesis and gliogenesis in the normal and damaged brain. Front Cell Neurosci 6, 70.

Cliffer, K., Burstein, R., Giesler, G., 1988. Distribution in the hypothalamus and telencephalon of fibers originating in the spinal cord. Soc Neurosci Abs 14, 121.

Collins, T., Young, G. T., Millar, N. S., 2011. Competitive binding at a nicotinic receptor transmembrane site of two alpha7-selective positive allosteric modulators with differing effects on agonist-evoked desensitization. Neuropharmacology 61, 1306-1313.

Colton, C., Wilcock, D. M., 2010. Assessing activation states in microglia. CNS Neurol Disord Drug Targets 9, 174-191.

Conejero-Goldberg, C., Davies, P., Ulloa, L., 2008. Alpha7 nicotinic acetylcholine receptor: a link between inflammation and neurodegeneration. Neurosci Biobehav Rev 32, 693-706.

Corringer, P. J., Le Novere, N., Changeux, J. P., 2000. Nicotinic receptors at the amino acid level. Annu Rev Pharmacol Toxicol 40, 431-458.

Coull, J. A., Beggs, S., Boudreau, D., Boivin, D., Tsuda, M., Inoue, K., Gravel, C., Salter, M. W., De Koninck, Y., 2005. BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. Nature 438, 1017-1021.

Coull, J. A., Boudreau, D., Bachand, K., Prescott, S. A., Nault, F., Sik, A., De Koninck, P., De Koninck, Y., 2003. Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. Nature 424, 938-942.

Couturier, S., Bertrand, D., Matter, J. M., Hernandez, M. C., Bertrand, S., Millar, N., Valera, S., Barkas, T., Ballivet, M., 1990. A neuronal nicotinic acetylcholine receptor subunit (alpha 7) is developmentally regulated and forms a homo-oligomeric channel blocked by alpha-BTX. Neuron 5, 847-856.

Covey, W. C., Ignatowski, T. A., Knight, P. R., Spengler, R. N., 2000. Brain-derived TNFalpha: involvement in neuroplastic changes implicated in the conscious perception of persistent pain. Brain Res 859, 113-122.

Covey, W. C., Ignatowski, T. A., Renauld, A. E., Knight, P. R., Nader, N. D., Spengler, R. N., 2002. Expression of neuron-associated tumor necrosis factor alpha in the brain is increased during persistent pain. Reg Anesth Pain Med 27, 357-366.

Cragg, J. J., Scott, A. L., Ramer, M. S., 2010. Depletion of spinal 5-HT accelerates mechanosensory recovery in the deafferented rat spinal cord. Exp Neurol 222, 277-284.

Cramer, S. W., Baggott, C., Cain, J., Tilghman, J., Allcock, B., Miranpuri, G., Rajpal, S., Sun, D., Resnick, D., 2008. The role of cation-dependent chloride transporters in neuropathic pain following spinal cord injury. Mol Pain 4, 36.

Cuellar, J. M., Montesano, P. X., Carstens, E., 2004. Role of TNF-alpha in sensitization of nociceptive dorsal horn neurons induced by application of nucleus pulposus to L5 dorsal root ganglion in rats. Pain 110, 578-587.

Dajas-Bailador, F., Wonnacott, S., 2004. Nicotinic acetylcholine receptors and the regulation of neuronal signalling. Trends Pharmacol Sci 25, 317-324.

Damaj, M. I., Meyer, E. M., Martin, B. R., 2000. The antinociceptive effects of alpha7 nicotinic agonists in an acute pain model. Neuropharmacology 39, 2785-2791.

Darman, R. B., Forbush, B., 2002. A regulatory locus of phosphorylation in the N terminus of the Na-K-Cl cotransporter, NKCC1. J Biol Chem 277, 37542-37550.

Davies, A. R., Hardick, D. J., Blagbrough, I. S., Potter, B. V., Wolstenholme, A. J., Wonnacott, S., 1999. Characterisation of the binding of [3H]methyllycaconitine: a new radioligand for labelling alpha 7-type neuronal nicotinic acetylcholine receptors. Neuropharmacology 38, 679-690.

de Jonge, W. J., Ulloa, L., 2007. The alpha7 nicotinic acetylcholine receptor as a pharmacological target for inflammation. Br J Pharmacol 151, 915-929.

De Koninck, Y., 2007. Altered chloride homeostasis in neurological disorders: a new target. Curr Opin Pharmacol 7, 93-99.

de Lucas-Cerrillo, A. M., Maldifassi, M. C., Arnalich, F., Renart, J., Atienza, G., Serantes, R., Cruces, J., Sanchez-Pacheco, A., Andres-Mateos, E., Montiel, C., 2011. Function of partially duplicated human α77 nicotinic receptor subunit CHRFAM7A gene: potential implications for the cholinergic anti-inflammatory response. J Biol Chem 286, 594-606.

del Rey, A., Yau, H. J., Randolf, A., Centeno, M. V., Wildmann, J., Martina, M., Besedovsky, H. O., Apkarian, A. V., 2011. Chronic neuropathic pain-like behavior correlates with IL-1beta expression and disrupts cytokine interactions in the hippocampus. Pain 152, 2827-2835.

DeLeo, J. A., Tanga, F. Y., Tawfik, V. L., 2004. Neuroimmune activation and neuroinflammation in chronic pain and opioid tolerance/hyperalgesia. Neuroscientist 10, 40-52.

Delpire, E., Mount, D. B., 2002. Human and murine phenotypes associated with defects in cation-chloride cotransport. Annu Rev Physiol 64, 803-843.

Descarries, L., Gisiger, V., Steriade, M., 1997. Diffuse transmission by acetylcholine in the CNS. Prog Neurobiol 53, 603-625.

DiDonato, J., Mercurio, F., Rosette, C., Wu-Li, J., Suyang, H., Ghosh, S., Karin, M., 1996. Mapping of the inducible IkappaB phosphorylation sites that signal its ubiquitination and degradation. Mol Cell Biol 16, 1295-1304.

Dineley, K. T., Pandya, A. A., Yakel, J. L., 2015. Nicotinic ACh receptors as therapeutic targets in CNS disorders. Trends Pharmacol Sci 36, 96-108.

Dixon, W. J., 1980. Efficient analysis of experimental observations. Annu Rev Pharmacol Toxicol 20, 441-462.

Dixon, W. R., Mosimann, W. F., Weiner, N., 1979. The role of presynatpic feedback mechanisms in regulation of norepinephrine release by nerve stimulation. J Pharmacol Exp Ther 209, 196-204.

Drago, J., McColl, C. D., Horne, M. K., Finkelstein, D. I., Ross, S. A., 2003. Neuronal nicotinic receptors: insights gained from gene knockout and knockin mutant mice. Cell Mol Life Sci 60, 1267-1280.

Drisdel, R. C., Green, W. N., 2000. Neuronal alpha-bungarotoxin receptors are alpha7 subunit homomers. J Neurosci 20, 133-139.

Duric, V., McCarson, K. E., 2006. Persistent pain produces stress-like alterations in hippocampal neurogenesis and gene expression. J Pain 7, 544-555.

Duric, V., McCarson, K. E., 2007. Neurokinin-1 (NK-1) receptor and brain-derived neurotrophic factor (BDNF) gene expression is differentially modulated in the rat spinal dorsal horn and hippocampus during inflammatory pain. Mol Pain 3, 32.

Dutar, P., Lamour, Y., Jobert, A., 1985. Activation of identified septo-hippocampal neurons by noxious peripheral stimulation. Brain Res 328, 15-21.

Dworkin, R. H., O'Connor, A. B., Backonja, M., Farrar, J. T., Finnerup, N. B., Jensen, T. S., Kalso, E. A., Loeser, J. D., Miaskowski, C., Nurmikko, T. J., Portenoy, R. K., Rice, A. S., Stacey, B. R., Treede, R. D., Turk, D. C., Wallace, M. S., 2007. Pharmacologic management of neuropathic pain: evidence-based recommendations. Pain 132, 237-251.

Egea, J., Buendia, I., Parada, E., Navarro, E., Leon, R., Lopez, M. G., 2015. Anti-inflammatory role of microglial alpha7 nAChRs and its role in neuroprotection. Biochem Pharmacol 97, 463-472.

Ehlers, M. R., 2000. CR3: a general purpose adhesion-recognition receptor essential for innate immunity. Microbes Infect 2, 289-294.

Ellis, A., Bennett, D., 2013. Neuroinflammation and the generation of neuropathic pain. Br J Anaesth 111, 26-37.

Ellis, A., Wieseler, J., Favret, J., Johnson, K. W., Rice, K. C., Maier, S. F., Falci, S., Watkins, L. R., 2014. Systemic administration of propentofylline, ibudilast, and (+)-naltrexone each reverses mechanical allodynia in a novel rat model of central neuropathic pain. J Pain 15, 407-421.

Ernfors, P., Ibanez, C. F., Ebendal, T., Olson, L., Persson, H., 1990. Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: developmental and topographical expression in the brain. Proc Natl Acad Sci U S A 87, 5454-5458.

Farooqui, A. A., Horrocks, L. A., 1994. Excitotoxicity and neurological disorders: involvement of membrane phospholipids. Int Rev Neurobiol 36, 267-323.

Ferrini, F., De Koninck, Y., 2013. Microglia control neuronal network excitability via BDNF signalling. Neural Plast 2013, 429815.

Feuerbach, D., Lingenhoehl, K., Olpe, H. R., Vassout, A., Gentsch, C., Chaperon, F., Nozulak, J., Enz, A., Bilbe, G., McAllister, K., Hoyer, D., 2009. The selective nicotinic acetylcholine receptor alpha7 agonist JN403 is active in animal models of cognition, sensory gating, epilepsy and pain. Neuropharmacology 56, 254-263.

Fong, J. C., Benamour, K., Szymonski, B., Thomasson, F., MORAND, J.-M., Cussac, M., 2000. Synthesis and. ALPHA.-Adrenergic Binding Ligand Affinities of 2-Iminoimidazolidine Derivatives. Chem Pharm Bull(Tokyo) 48, 729-733.

Freitas, K., Negus, S. S., Carroll, F. I., Damaj, M. I., 2013. In vivo pharmacological interactions between a type II positive allosteric modulator of alpha7 nicotinic ACh receptors and nicotinic agonists in a murine tonic pain model. Br J Pharmacol 169, 567-579.

Fucile, S., 2004. Ca2+ permeability of nicotinic acetylcholine receptors. Cell Calcium 35, 1-8.

Gahring, L. C., Rogers, S. W., 2005. Neuronal nicotinic acetylcholine receptor expression and function on nonneuronal cells. AAPS J 7, E885-E894.

Gaimarri, A., Moretti, M., Riganti, L., Zanardi, A., Clementi, F., Gotti, C., 2007. Regulation of neuronal nicotinic receptor traffic and expression. Brain Res Rev 55, 134-143.

Galan, A., Cervero, F., 2005. Painful stimuli induce in vivo phosphorylation and membrane mobilization of mouse spinal cord NKCC1 co-transporter. Neuroscience 133, 245-252.

Gao, Y., Lecker, S., Post, M. J., Hietaranta, A. J., Li, J., Volk, R., Li, M., Sato, K., Saluja, A. K., Steer, M. L., Goldberg, A. L., Simons, M., 2000. Inhibition of ubiquitin-proteasome pathway-mediated I kappa B alpha degradation by a naturally occurring antibacterial peptide. J Clin Invest 106, 439-448.

Garraffo, H. M., Spande, T. F., Williams, M., 2009. Epibatidine: from frog alkaloid to analgesic clinical candidates. A Testimonial to "true grit"! Heterocycles 79, 207-217.

Garrison, C. J., Dougherty, P. M., Kajander, K. C., Carlton, S. M., 1991. Staining of glial fibrillary acidic protein (GFAP) in lumbar spinal cord increases following a sciatic nerve constriction injury. Brain Res 565, 1-7.

Gerard, E., Spengler, R. N., Bonoiu, A. C., Mahajan, S. D., Davidson, B. A., Ding, H., Kumar, R., Prasad, P. N., Knight, P. R., Ignatowski, T. A., 2015. Chronic constriction injury-induced nociception is relieved by nanomedicine-mediated decrease of rat hippocampal tumor necrosis factor. Pain 156, 1320-1333.

Giebelen, I. A., van Westerloo, D. J., LaRosa, G. J., de Vos, A. F., van der Poll, T., 2007a. Local stimulation of alpha7 cholinergic receptors inhibits LPS-induced TNF-alpha release in the mouse lung. Shock 28, 700-703.

Giebelen, I. A., van Westerloo, D. J., LaRosa, G. J., de Vos, A. F., van der Poll, T., 2007b. Stimulation of alpha 7 cholinergic receptors inhibits lipopolysaccharide-induced neutrophil recruitment by a tumor necrosis factor alpha-independent mechanism. Shock 27, 443-447.

Gill, J. K., Dhankher, P., Sheppard, T. D., Sher, E., Millar, N. S., 2012. A series of alpha7 nicotinic acetylcholine receptor allosteric modulators with close chemical similarity but diverse pharmacological properties. Mol Pharmacol 81, 710-718.

Gill, J. K., Savolainen, M., Young, G. T., Zwart, R., Sher, E., Millar, N. S., 2011. Agonist activation of alpha7 nicotinic acetylcholine receptors via an allosteric transmembrane site. Proc Natl Acad Sci U S A 108, 5867-5872.

Gomes, C., Ferreira, R., George, J., Sanches, R., Rodrigues, D. I., Goncalves, N., Cunha, R. A., 2013. Activation of microglial cells triggers a release of brain-derived neurotrophic factor (BDNF) inducing their proliferation in an adenosine A2A receptor-dependent manner: A2A receptor blockade prevents BDNF release and proliferation of microglia. J Neuroinflammation 10, 16.

Gonzalez-Scarano, F., Baltuch, G., 1999. Microglia as mediators of inflammatory and degenerative diseases. Annu Rev Neurosci 22, 219-240.

Gotti, C., Clementi, F., 2004. Neuronal nicotinic receptors: from structure to pathology. Prog Neurobiol 74, 363-396.

Gotti, C., Moretti, M., Gaimarri, A., Zanardi, A., Clementi, F., Zoli, M., 2007. Heterogeneity and complexity of native brain nicotinic receptors. Biochem Pharmacol 74, 1102-1111.

Gotti, C., Zoli, M., Clementi, F., 2006. Brain nicotinic acetylcholine receptors: native subtypes and their relevance. Trends Pharmacol Sci 27, 482-491.

Graeber, M. B., 2010. Changing face of microglia. Science 330, 783-788.

Graeber, M. B., Streit, W. J., 2010. Microglia: biology and pathology. Acta Neuropathol 119, 89-105.

Gronlien, J. H., Hakerud, M., Ween, H., Thorin-Hagene, K., Briggs, C. A., Gopalakrishnan, M., Malysz, J., 2007. Distinct profiles of alpha7 nAChR positive allosteric modulation revealed by structurally diverse chemotypes. Mol Pharmacol 72, 715-724.

Groth, R., Aanonsen, L., 2002. Spinal brain-derived neurotrophic factor (BDNF) produces hyperalgesia in normal mice while antisense directed against either BDNF or trkB, prevent inflammation-induced hyperalgesia. Pain 100, 171-181.

Grybko, M., Sharma, G., Vijayaraghavan, S., 2010. Functional distribution of nicotinic receptors in CA3 region of the hippocampus. J Mol Neurosci 40, 114-120.

Guo, L. H., Schluesener, H. J., 2006. Acute but not chronic stimulation of glial cells in rat spinal cord by systemic injection of lipopolysaccharide is associated with hyperalgesia. Acta Neuropathol 112, 703-713.

Guo, W., Robbins, M. T., Wei, F., Zou, S., Dubner, R., Ren, K., 2006. Supraspinal brain-derived neurotrophic factor signaling: a novel mechanism for descending pain facilitation. J Neurosci 26, 126-137.

Haas, M., 1994. The Na-K-Cl cotransporters. Am J Physiol 267, C869-885.

Hains, B. C., Waxman, S. G., 2006. Activated microglia contribute to the maintenance of chronic pain after spinal cord injury. J Neurosci 26, 4308-4317.

Han, J. S., Neugebauer, V., 2005. mGluR1 and mGluR5 antagonists in the amygdala inhibit different components of audible and ultrasonic vocalizations in a model of arthritic pain. Pain 113, 211-222.

Haraguchi, T., Iwasaki, K., Takasaki, K., Uchida, K., Naito, T., Nogami, A., Kubota, K., Shindo, T., Uchida, N., Katsurabayashi, S., Mishima, K., Nishimura, R., Fujiwara, M., 2010. Telmisartan, a partial agonist of peroxisome proliferator-activated receptor gamma, improves impairment of spatial memory and hippocampal apoptosis in rats treated with repeated cerebral ischemia. Brain Res 1353, 125-132.

Haydar, S. N., Dunlop, J., 2010. Neuronal nicotinic acetylcholine receptors-targets for the development of drugs to treat cognitive impairment associated with schizophrenia and Alzheimer's disease. Curr Top Med Chem 10, 144-152.

Hogg, R. C., Bertrand, D., 2004. Nicotinic acetylcholine receptors as drug targets. Curr Drug Targets CNS Neurol Disord 3, 123-130.

Hogg, R. C., Raggenbass, M., Bertrand, D., 2003. Nicotinic acetylcholine receptors: from structure to brain function. Rev Physiol Biochem Pharmacol 147, 1-46.

Holladay, M. W., Dart, M. J., Lynch, J. K., 1997. Neuronal nicotinic acetylcholine receptors as targets for drug discovery. J Med Chem 40, 4169-4194.

Homma, Y., Brull, S. J., Zhang, J. M., 2002. A comparison of chronic pain behavior following local application of tumor necrosis factor alpha to the normal and mechanically compressed lumbar ganglia in the rat. Pain 95, 239-246.

Hu, Y., Liu, R., Li, J., Yue, Y., Cheng, W., Zhang, P., 2014. Attenuation of Collagen-Induced Arthritis in rat by nicotinic alpha7 receptor partial agonist GTS-21. Biomed Res Int 2014, 325875.

Hua, S., Ek, C. J., Mallard, C., Johansson, M. E., 2014. Perinatal hypoxia-ischemia reduces alpha 7 nicotinic receptor expression and selective alpha 7 nicotinic receptor stimulation suppresses inflammation and promotes microglial Mox phenotype. Biomed Res Int 2014, 718769.

Huang, E. J., Reichardt, L. F., 2003. Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem 72, 609-642.

Hughes, I. W., Lustman, F., 1990. Bumetanide/amiloride ('Burinex A'): a new combined preparation for the control of patients with congestive cardiac failure. Curr Med Res Opin 12, 151-159.

Hurst, R., Rollema, H., Bertrand, D., 2013. Nicotinic acetylcholine receptors: from basic science to therapeutics. Pharmacol Ther 137, 22-54.

Hurst, R. S., Hajos, M., Raggenbass, M., Wall, T. M., Higdon, N. R., Lawson, J. A., Rutherford-Root, K. L., Berkenpas, M. B., Hoffmann, W. E., Piotrowski, D. W., Groppi, V. E., Allaman, G., Ogier, R., Bertrand, S., Bertrand, D., Arneric, S. P., 2005. A novel positive allosteric modulator of the alpha7 neuronal nicotinic acetylcholine receptor: in vitro and in vivo characterization. J Neurosci 25, 4396-4405.

Ignatowski, T. A., Covey, W. C., Knight, P. R., Severin, C. M., Nickola, T. J., Spengler, R. N., 1999. Brain-derived TNFalpha mediates neuropathic pain. Brain Res 841, 70-77.

Ignatowski, T. A., Spengler, R. N., 1994. Tumor necrosis factor-alpha: presynaptic sensitivity is modified after antidepressant drug administration. Brain Res 665, 293-299.

Ignatowski, T. A., Sud, R., Reynolds, J. L., Knight, P. R., Spengler, R. N., 2005. The dissipation of neuropathic pain paradoxically involves the presence of tumor necrosis factor-alpha (TNF). Neuropharmacology 48, 448-460.

Imai, Y., Ibata, I., Ito, D., Ohsawa, K., Kohsaka, S., 1996. A novel gene iba1 in the major histocompatibility complex class III region encoding an EF hand protein expressed in a monocytic lineage. Biochem Biophys Res Commun 224, 855-862.

Jaggi, A. S., Singh, N., 2011. Exploring the potential of telmisartan in chronic constriction injury-induced neuropathic pain in rats. Eur J Pharmacol 667, 215-221.

Jensen, A. A., Frolund, B., Liljefors, T., Krogsgaard-Larsen, P., 2005. Neuronal nicotinic acetylcholine receptors: structural revelations, target identifications, and therapeutic inspirations. J Med Chem 48, 4705-4745.

Jha, M. K., Jeon, S., Suk, K., 2012. Glia as a link between neuroinflammation and neuropathic pain. Immune Netw 12, 41-47.

Ji, G., Sun, H., Fu, Y., Li, Z., Pais-Vieira, M., Galhardo, V., Neugebauer, V., 2010. Cognitive impairment in pain through amygdala-driven prefrontal cortical deactivation. J Neurosci 30, 5451-5464.

Ji, R. R., Suter, M. R., 2007. p38 MAPK, microglial signaling, and neuropathic pain. Mol Pain 3, 33.

Ji, R. R., Wen, Y. R., 2006. Neural-glial interaction in the spinal cord for the development and maintenance of nerve injury-induced neuropathic pain. Drug Dev Res 67, 331-338.

Ji, R. R., Woolf, C. J., 2001. Neuronal plasticity and signal transduction in nociceptive neurons: implications for the initiation and maintenance of pathological pain. Neurobiol Dis 8, 1-10.

Jope, R. S., Gu, X., 1991. Seizures increase acetylcholine and choline concentrations in rat brain regions. Neurochem Res 16, 1219-1226.

Junger, H., Sorkin, L. S., 2000. Nociceptive and inflammatory effects of subcutaneous TNFalpha. Pain 85, 145-151.

Kabbani, N., Nordman, J. C., Corgiat, B. A., Veltri, D. P., Shehu, A., Seymour, V. A., Adams, D. J., 2013. Are nicotinic acetylcholine receptors coupled to G proteins? Bioessays 35, 1025-1034.

Kageyama-Yahara, N., Suehiro, Y., Yamamoto, T., Kadowaki, M., 2008. IgE-induced degranulation of mucosal mast cells is negatively regulated via nicotinic acetylcholine receptors. Biochem Biophys Res Commun 377, 321-325.

Kahle, K. T., Staley, K. J., 2008. The bumetanide-sensitive Na-K-2Cl cotransporter NKCC1 as a potential target of a novel mechanism-based treatment strategy for neonatal seizures. Neurosurg Focus 25, E22.

Kaila, K., 1994. Ionic basis of GABAA receptor channel function in the nervous system. Prog Neurobiol 42, 489-537.

Kapadia, R., Yi, J.-H., Vemuganti, R., 2008. Mechanisms of anti-inflammatory and neuroprotective actions of PPAR-gamma agonists. Front Biosci 13, 1813-1826.

Kawashima, K., Fujii, T., Moriwaki, Y., Misawa, H., Horiguchi, K., 2012. Reconciling neuronally and nonneuronally derived acetylcholine in the regulation of immune function. Ann N Y Acad Sci 1261, 7-17.

Kettenmann, H., Hanisch, U. K., Noda, M., Verkhratsky, A., 2011. Physiology of microglia. Physiol Rev 91, 461-553.

Khanna, S., Sinclair, J. G., 1989. Noxious stimuli produce prolonged changes in the CA1 region of the rat hippocampus. Pain 39, 337-343.

King, J. R., Nordman, J. C., Bridges, S. P., Lin, M. K., Kabbani, N., 2015. Identification and Characterization of a G Protein-binding Cluster in alpha7 Nicotinic Acetylcholine Receptors. J Biol Chem 290, 20060-20070.

Kiss, J. P., Zsilla, G., Mike, A., Zelles, T., Toth, E., Lajtha, A., Vizi, E. S., 1995. Subtype-specificity of the presynaptic alpha 2-adrenoceptors modulating hippocampal norepinephrine release in rat. Brain Res 674, 238-244.

Klein, J., Chatterjee, S. S., Loffelholz, K., 1997. Phospholipid breakdown and choline release under hypoxic conditions: inhibition by bilobalide, a constituent of Ginkgo biloba. Brain Res. 755, 347-350.

Klein, J. D., Lamitina, S. T., O'Neill, W. C., 1999. JNK is a volume-sensitive kinase that phosphorylates the Na-K-2Cl cotransporter in vitro. Am J Physiol 277, C425-C431.

Ko, M. C., Lee, M. C., Amstislavskaya, T. G., Tikhonova, M. A., Yang, Y. L., Lu, K. T., 2014. Inhibition of NKCC1 attenuated hippocampal LTP formation and inhibitory avoidance in rat. PLoS One 9, e106692.

Kodama, D., Ono, H., Tanabe, M., 2007. Altered hippocampal long-term potentiation after peripheral nerve injury in mice. Eur J Pharmacol 574, 127-132.

Kox, M., van Velzen, J. F., Pompe, J. C., Hoedemaekers, C. W., van der Hoeven, J. G., Pickkers, P., 2009. GTS-21 inhibits pro-inflammatory cytokine release independent of the Toll-like receptor stimulated via a transcriptional mechanism involving JAK2 activation. Biochem Pharmacol 78, 863-872.

Kreutzberg, G. W., 1996. Microglia: a sensor for pathological events in the CNS. Trends Neurosci 19, 312-318.

Kriegstein, A., Alvarez-Buylla, A., 2009. The glial nature of embryonic and adult neural stem cells. Annu Rev Neurosci 32, 149-184.

Laird, J. M., Garcia-Nicas, E., Delpire, E. J., Cervero, F., 2004. Presynaptic inhibition and spinal pain processing in mice: a possible role of the NKCC1 cation-chloride co-transporter in hyperalgesia. Neurosci Lett 361, 200-203.

Lawson, L. J., Perry, V. H., Dri, P., Gordon, S., 1990. Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. Neuroscience 39, 151-170.

Le Novere, N., Corringer, P. J., Changeux, J. P., 2002. The diversity of subunit composition in nAChRs: evolutionary origins, physiologic and pharmacologic consequences. J Neurobiol 53, 447-456.

Ledeboer, A., Gamanos, M., Lai, W., Martin, D., Maier, S. F., Watkins, L. R., Quan, N., 2005. Involvement of spinal cord nuclear factor kappaB activation in rat models of proinflammatory cytokine-mediated pain facilitation. Eur J Neurosci 22, 1977-1986.

Ledeboer, A., Hutchinson, M. R., Watkins, L. R., Johnson, K. W., 2007. Ibudilast (AV-411). A new class therapeutic candidate for neuropathic pain and opioid withdrawal syndromes. Expert Opin Investig Drugs 16, 935-950.

Ledeboer, A., Liu, T., Shumilla, J. A., Mahoney, J. H., Vijay, S., Gross, M. I., Vargas, J. A., Sultzbaugh, L., Claypool, M. D., Sanftner, L. M., Watkins, L. R., Johnson, K. W., 2006. The glial modulatory drug AV411 attenuates mechanical allodynia in rat models of neuropathic pain. Neuron Glia Biol 2, 279-291.

Lee, H. K., Ahmed, M. M., King, K. C., Miranpuri, G. S., Kahle, K. T., Resnick, D. K., Sun, D., 2014. Persistent phosphorylation of NKCC1 and WNK1 in the epicenter of the spinal cord following contusion injury. Spine J 14, 777-781.

Lee, S., Zhao, Y. Q., Ribeiro-da-Silva, A., Zhang, J., 2010. Distinctive response of CNS glial cells in oro-facial pain associated with injury, infection and inflammation. Mol Pain 6, 79.

Li, L., Xian, C. J., Zhong, J. H., Zhou, X. F., 2006. Upregulation of brain-derived neurotrophic factor in the sensory pathway by selective motor nerve injury in adult rats. Neurotox Res 9, 269-283.

Li, Q., Verma, I. M., 2002. NF-kappaB regulation in the immune system. Nat Rev.Immunol 2, 725-734.

Li, X. Y., Ko, H. G., Chen, T., Descalzi, G., Koga, K., Wang, H., Kim, S. S., Shang, Y., Kwak, C., Park, S. W., Shim, J., Lee, K., Collingridge, G. L., Kaang, B. K., Zhuo, M., 2010. Alleviating neuropathic pain hypersensitivity by inhibiting PKMzeta in the anterior cingulate cortex. Science 330, 1400-1404.

Ling, E. A., Wong, W. C., 1993. The origin and nature of ramified and amoeboid microglia: a historical review and current concepts. Glia 7, 9-18.

Loram, L. C., Harrison, J. A., Chao, L., Taylor, F. R., Reddy, A., Travis, C. L., Giffard, R., Al-Abed, Y., Tracey, K., Maier, S. F., Watkins, L. R., 2010. Intrathecal injection of an alpha seven nicotinic acetylcholine receptor agonist attenuates gp120-induced mechanical allodynia and spinal pro-inflammatory cytokine profiles in rats. Brain Behav Immun 24, 959-967.

Loram, L. C., Taylor, F. R., Strand, K. A., Maier, S. F., Speake, J. D., Jordan, K. G., James, J. W., Wene, S. P., Pritchard, R. C., Green, H., Van Dyke, K., Mazarov, A., Letchworth, S. R., Watkins, L. R., 2012. Systemic administration of an alpha-7 nicotinic acetylcholine agonist reverses neuropathic pain in male Sprague Dawley rats. J Pain 13, 1162-1171.

Lukas, R. J., Changeux, J. P., Le Novere, N., Albuquerque, E. X., Balfour, D. J., Berg, D. K., Bertrand, D., Chiappinelli, V. A., Clarke, P. B., Collins, A. C., Dani, J. A., Grady, S. R., Kellar, K. J., Lindstrom, J. M., Marks, M. J., Quik, M., Taylor, P. W., Wonnacott, S., 1999. International Union of Pharmacology. XX. Current status of the nomenclature for nicotinic acetylcholine receptors and their subunits. Pharmacol Rev 51, 397-401.

Ma, W., Bisby, M. A., 1998. Increased activation of nuclear factor kappa B in rat lumbar dorsal root ganglion neurons following partial sciatic nerve injuries. Brain Res 797, 243-254.

MacEwan, D. J., 2002. TNF receptor subtype signalling: differences and cellular consequences. Cell Signal 14, 477-492.

Mannion, R. J., Costigan, M., Decosterd, I., Amaya, F., Ma, Q. P., Holstege, J. C., Ji, R. R., Acheson, A., Lindsay, R. M., Wilkinson, G. A., Woolf, C. J., 1999. Neurotrophins: peripherally and centrally acting modulators of tactile stimulus-induced inflammatory pain hypersensitivity. Proc Natl Acad Sci U S A 96, 9385-9390.

Marchand, F., Perretti, M., McMahon, S. B., 2005. Role of the immune system in chronic pain. Nat Rev Neurosci 6, 521-532.

Martuscello, R. T., Spengler, R. N., Bonoiu, A. C., Davidson, B. A., Helinski, J., Ding, H., Mahajan, S., Kumar, R., Bergey, E. J., Knight, P. R., Prasad, P. N., Ignatowski, T. A.,

2012. Increasing TNF levels solely in the rat hippocampus produces persistent pain-like symptoms. Pain 153, 1871-1882.

Mazurov, A. A., Speake, J. D., Yohannes, D., 2011. Discovery and development of alpha7 nicotinic acetylcholine receptor modulators. J Med Chem 54, 7943-7961.

Medhurst, S. J., Hatcher, J. P., Hille, C. J., Bingham, S., Clayton, N. M., Billinton, A., Chessell, I. P., 2008. Activation of the alpha7-nicotinic acetylcholine receptor reverses complete freund adjuvant-induced mechanical hyperalgesia in the rat via a central site of action. J Pain 9, 580-587.

Meller, S. T., Dykstra, C., Grzybycki, D., Murphy, S., Gebhart, G. F., 1994. The possible role of glia in nociceptive processing and hyperalgesia in the spinal cord of the rat. Neuropharmacology 33, 1471-1478.

Mencel, M., Nash, M., Jacobson, C., 2013. Neuregulin upregulates microglial alpha7 nicotinic acetylcholine receptor expression in immortalized cell lines: implications for regulating neuroinflammation. PLoS One 8, e70338.

Metz, A. E., Yau, H. J., Centeno, M. V., Apkarian, A. V., Martina, M., 2009. Morphological and functional reorganization of rat medial prefrontal cortex in neuropathic pain. Proc Natl Acad Sci U S A 106, 2423-2428.

Mika, J., 2008. Modulation of microglia can attenuate neuropathic pain symptoms and enhance morphine effectiveness. Pharmacol Rep 60, 297-307.

Mika, J., Zychowska, M., Popiolek-Barczyk, K., Rojewska, E., Przewlocka, B., 2013. Importance of glial activation in neuropathic pain. Eur J Pharmacol 716, 106-119.

Millan, M. J., 2002. Descending control of pain. Prog Neurobiol 66, 355-474.

Milligan, E. D., Mehmert, K. K., Hinde, J. L., Harvey, L. O., Martin, D., Tracey, K. J., Maier, S. F., Watkins, L. R., 2000. Thermal hyperalgesia and mechanical allodynia produced by intrathecal administration of the human immunodeficiency virus-1 (HIV-1) envelope glycoprotein, gp120. Brain Res 861, 105-116.

Milligan, E. D., O'Connor, K. A., Nguyen, K. T., Armstrong, C. B., Twining, C., Gaykema, R. P., Holguin, A., Martin, D., Maier, S. F., Watkins, L. R., 2001. Intrathecal HIV-1 envelope glycoprotein gp120 induces enhanced pain states mediated by spinal cord proinflammatory cytokines. J Neurosci 21, 2808-2819.

Milligan, E. D., Twining, C., Chacur, M., Biedenkapp, J., O'Connor, K., Poole, S., Tracey, K., Martin, D., Maier, S. F., Watkins, L. R., 2003. Spinal glia and proinflammatory cytokines mediate mirror-image neuropathic pain in rats. J Neurosci 23, 1026-1040.

Milligan, E. D., Zapata, V., Chacur, M., Schoeniger, D., Biedenkapp, J., O'Connor, K. A., Verge, G. M., Chapman, G., Green, P., Foster, A. C., Naeve, G. S., Maier, S. F., Watkins, L. R., 2004. Evidence that exogenous and endogenous fractalkine can induce spinal nociceptive facilitation in rats. Eur J Neurosci 20, 2294-2302.

Miwa, T., Furukawa, S., Nakajima, K., Furukawa, Y., Kohsaka, S., 1997. Lipopolysaccharide enhances synthesis of brain-derived neurotrophic factor in cultured rat microglia. J Neurosci Res 50, 1023-1029.

Moalem, G., Tracey, D. J., 2006. Immune and inflammatory mechanisms in neuropathic pain. Brain Res Rev 51, 240-264.

Mori, I., Imai, Y., Kohsaka, S., Kimura, Y., 2000. Upregulated expression of Iba1 molecules in the central nervous system of mice in response to neurovirulent influenza A virus infection. Microbiol Immunol 44, 729-735.

Morioka, N., Harano, S., Tokuhara, M., Idenoshita, Y., Zhang, F. F., Hisaoka-Nakashima, K., Nakata, Y., 2015. Stimulation of alpha7 nicotinic acetylcholine receptor regulates glutamate transporter GLAST via basic fibroblast growth factor production in cultured cortical microglia. Brain Res 1625, 111-120.

Morioka, T., Kalehua, A. N., Streit, W. J., 1991. The microglial reaction in the rat dorsal hippocampus following transient forebrain ischemia. J Cereb Blood Flow Metab 11, 966-973.

Mulcahy, M. J., Blattman, S. B., Barrantes, F. J., Lukas, R. J., Hawrot, E., 2015. Resistance to Inhibitors of Cholinesterase 3 (Ric-3) Expression Promotes Selective Protein Associations with the Human alpha7-Nicotinic Acetylcholine Receptor Interactome. PLoS One 10, e0134409.

Munro, G., Hansen, R., Erichsen, H., Timmermann, D., Christensen, J., Hansen, H., 2012. The alpha7 nicotinic ACh receptor agonist compound B and positive allosteric modulator PNU-120596 both alleviate inflammatory hyperalgesia and cytokine release in the rat. Br J Pharmacol 167, 421-435.

Mutso, A. A., Radzicki, D., Baliki, M. N., Huang, L., Banisadr, G., Centeno, M. V., Radulovic, J., Martina, M., Miller, R. J., Apkarian, A. V., 2012. Abnormalities in hippocampal functioning with persistent pain. J Neurosci 32, 5747-5756.

Nakajima, K., Kohsaka, S., 2001. Microglia: activation and their significance in the central nervous system. J Biochem 130, 169-175.

Nomura, H., Sakai, A., Nagano, M., Umino, M., Suzuki, H., 2006. Expression changes of cation chloride cotransporters in the rat spinal cord following intraplantar formalin. Neurosci Res 56, 435-440.

Oka, T., Wakugawa, Y., Hosoi, M., Oka, K., Hori, T., 1996. Intracerebroventricular injection of tumor necrosis factor-alpha induces thermal hyperalgesia in rats. Neuroimmunomodulation 3, 135-140.

Pabreja, K., Dua, K., Sharma, S., Padi, S. S., Kulkarni, S. K., 2011. Minocycline attenuates the development of diabetic neuropathic pain: possible anti-inflammatory and anti-oxidant mechanisms. Eur J Pharmacol 661, 15-21.

Pacini, A., Di Cesare Mannelli, L., Bonaccini, L., Ronzoni, S., Bartolini, A., Ghelardini, C., 2010. Protective effect of alpha7 nAChR: behavioural and morphological features on neuropathy. Pain 150, 542-549.

Pahl, H. L., 1999. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 18, 6853-6866.

Palsson-McDermott, E. M., O'Neill, L. A., 2004. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. Immunology 113, 153-162.

Papke, R. L., Bagdas, D., Kulkarni, A. R., Gould, T., AlSharari, S. D., Thakur, G. A., Damaj, M. I., 2015. The analgesic-like properties of the alpha7 nAChR silent agonist NS6740 is associated with non-conducting conformations of the receptor. Neuropharmacology 91, 34-42.

Papke, R. L., Bencherif, M., Lippiello, P., 1996. An evaluation of neuronal nicotinic acetylcholine receptor activation by quaternary nitrogen compounds indicates that choline is selective for the α7 subtype. Neurosci Lett 213, 201-204.

Papke, R. L., Kem, W. R., Soti, F., Lopez-Hernandez, G. Y., Horenstein, N. A., 2009. Activation and desensitization of nicotinic alpha7-type acetylcholine receptors by benzylidene anabaseines and nicotine. J Pharmacol Exp Ther 329, 791-807.

Paulo, J. A., Brucker, W. J., Hawrot, E., 2009. Proteomic analysis of an alpha7 nicotinic acetylcholine receptor interactome. J Proteome Res 8, 1849-1858.

Pavlov, V. A., Ochani, M., Yang, L. H., Gallowitsch-Puerta, M., Ochani, K., Lin, X., Levi, J., Parrish, W. R., Rosas-Ballina, M., Czura, C. J., Larosa, G. J., Miller, E. J., Tracey, K. J., Al-Abed, Y., 2007. Selective alpha7-nicotinic acetylcholine receptor agonist GTS-21 improves survival in murine endotoxemia and severe sepsis. Crit Care Med 35, 1139-1144.

Pavlov, V. A., Tracey, K. J., 2006. Controlling inflammation: the cholinergic anti-inflammatory pathway. Biochem Soc Trans 34, 1037-1040.

Payne, J. A., Rivera, C., Voipio, J., Kaila, K., 2003. Cation-chloride co-transporters in neuronal communication, development and trauma. Trends Neurosci 26, 199-206.

Payne, J. A., Stevenson, T. J., Donaldson, L. F., 1996. Molecular characterization of a putative K-Cl cotransporter in rat brain. A neuronal-specific isoform. J Biol Chem 271, 16245-16252.

Pena, G., Cai, B., Liu, J., van der Zanden, E. P., Deitch, E. A., de Jonge, W. J., Ulloa, L., 2010. Unphosphorylated STAT3 modulates alpha 7 nicotinic receptor signaling and cytokine production in sepsis. Eur J Immunol 40, 2580-2589.

Pezet, S., Malcangio, M., 2004. Brain-derived neurotrophic factor as a drug target for CNS disorders. Expert Opin Ther Targets 8, 391-399.

Picciotto, M. R., Caldarone, B. J., Brunzell, D. H., Zachariou, V., Stevens, T. R., King, S. L., 2001. Neuronal nicotinic acetylcholine receptor subunit knockout mice: physiological and behavioral phenotypes and possible clinical implications. Pharmacol Ther 92, 89-108.

Picciotto, M. R., Caldarone, B. J., King, S. L., Zachariou, V., 2000. Nicotinic receptors in the brain. Links between molecular biology and behavior. Neuropsychopharmacology 22, 451-465.

Pitcher, M. H., Cervero, F., 2010. Role of the NKCC1 co-transporter in sensitization of spinal nociceptive neurons. Pain 151, 756-762.

Plotkin, M. D., Kaplan, M. R., Peterson, L. N., Gullans, S. R., Hebert, S. C., Delpire, E., 1997. Expression of the Na(+)-K(+)-2Cl- cotransporter BSC2 in the nervous system. Am J Physiol 272, C173-183.

Pocock, J. M., Kettenmann, H., 2007. Neurotransmitter receptors on microglia. Trends Neurosci 30, 527-535.

Price, T. J., Cervero, F., de Koninck, Y., 2005. Role of cation-chloride-cotransporters (CCC) in pain and hyperalgesia. Curr Top Med Chem 5, 547-555.

Price, T. J., Cervero, F., Gold, M. S., Hammond, D. L., Prescott, S. A., 2009. Chloride regulation in the pain pathway. Brain Res Rev 60, 149-170.

Qian, C., Li, T., Shen, T. Y., Libertine-Garahan, L., Eckman, J., Biftu, T., Ip, S., 1993. Epibatidine is a nicotinic analgesic. Eur J Pharmacol 250, R13-14.

Quintao, N. L., Santos, A. R., Campos, M. M., Calixto, J. B., 2008. The role of neurotrophic factors in genesis and maintenance of mechanical hypernociception after brachial plexus avulsion in mice. Pain 136, 125-133.

Remko, M., Swart, M., Bickelhaupt, F. M., 2006. Theoretical study of structure, pK a, lipophilicity, solubility, absorption, and polar surface area of some centrally acting antihypertensives. Bioorg Med Chem 14, 1715-1728.

Reynolds, J. L., Ignatowski, T. A., Gallant, S., Spengler, R. N., 2004. Amitriptyline administration transforms tumor necrosis factor-alpha regulation of norepinephrine release in the brain. Brain Res 1023, 112-120.

Rivera, C., Li, H., Thomas-Crusells, J., Lahtinen, H., Viitanen, T., Nanobashvili, A., Kokaia, Z., Airaksinen, M. S., Voipio, J., Kaila, K., Saarma, M., 2002. BDNF-induced TrkB activation down-regulates the K+-Cl- cotransporter KCC2 and impairs neuronal Cl- extrusion. J Cell Biol 159, 747-752.

Rivera, C., Voipio, J., Thomas-Crusells, J., Li, H., Emri, Z., Sipila, S., Payne, J. A., Minichiello, L., Saarma, M., Kaila, K., 2004. Mechanism of activity-dependent downregulation of the neuron-specific K-Cl cotransporter KCC2. J Neurosci 24, 4683-4691.

Rivest, S., 2003. Molecular insights on the cerebral innate immune system. Brain Behav Immun 17, 13-19.

Rock, R. B., Gekker, G., Hu, S., Sheng, W. S., Cheeran, M., Lokensgard, J. R., Peterson, P. K., 2004. Role of microglia in central nervous system infections. Clin Microbiol Rev 17, 942-964.

Romanelli, P., Esposito, V., 2004. The functional anatomy of neuropathic pain. Neurosurg Clin N Am 15, 257-268.

Romero-Sandoval, E. A., Horvath, R. J., DeLeo, J. A., 2008. Neuroimmune interactions and pain: focus on glial-modulating targets. Curr Opin Investig Drugs 9, 726-734.

Roncarati, R., Seredenina, T., Jow, B., Jow, F., Papini, S., Kramer, A., Bothmann, H., Dunlop, J., Terstappen, G. C., 2008a. Functional properties of alpha7 nicotinic acetylcholine receptors co-expressed with RIC-3 in a stable recombinant CHO-K1 cell line. Assay Drug Dev. Technol. 6, 181-193.

Roncarati, R., Seredenina, T., Jow, B., Jow, F., Papini, S., Kramer, A., Bothmann, H., Dunlop, J., Terstappen, G. C., 2008b. Functional Properties of α 7 Nicotinic Acetylcholine Receptors Co-expressed with RIC-3 in a Stable Recombinant CHO-K1 Cell Line. Assay Drug Dev Technol 6, 181-193.

Roni, M. A., Rahman, S., 2013. Antidepressant-like effects of lobeline in mice: behavioral, neurochemical, and neuroendocrine evidence. Prog Neuropsychopharmacol Biol Psychiatry 41, 44-51.

Rosas-Ballina, M., Goldstein, R. S., Gallowitsch-Puerta, M., Yang, L., Valdes-Ferrer, S. I., Patel, N. B., Chavan, S., Al-Abed, Y., Yang, H., Tracey, K. J., 2009. The selective alpha7 agonist GTS-21 attenuates cytokine production in human whole blood and human monocytes activated by ligands for TLR2, TLR3, TLR4, TLR9, and RAGE. Mol Med 15, 195-202.

Rowley, T. J., McKinstry, A., Greenidge, E., Smith, W., Flood, P., 2010. Antinociceptive and anti-inflammatory effects of choline in a mouse model of postoperative pain. Br J Anaesth 105, 201-207.

Rowley, T. J., Payappilly, J., Lu, J., Flood, P., 2008. The antinociceptive response to nicotinic agonists in a mouse model of postoperative pain. Anesth Analg 107, 1052-1057.

Saavedra, J. M., 2012. Angiotensin II AT(1) receptor blockers as treatments for inflammatory brain disorders. Clin Sci (Lond) 123, 567-590.

Sahley, T. L., Berntson, G. G., 1979. Antinociceptive effects of central and systemic administrations of nicotine in the rat. Psychopharmacology (Berl) 65, 279-283.

Schafers, M., Geis, C., Svensson, C. I., Luo, Z. D., Sommer, C., 2003. Selective increase of tumour necrosis factor-alpha in injured and spared myelinated primary afferents after chronic constrictive injury of rat sciatic nerve. Eur J Neurosci 17, 791-804.

Scheinin, M., Lomasney, J. W., Hayden-Hixson, D. M., Schambra, U. B., Caron, M. G., Lefkowitz, R. J., Fremeau, R. T., Jr., 1994. Distribution of alpha 2-adrenergic receptor subtype gene expression in rat brain. Brain Res Mol Brain Res 21, 133-149.

Scholz, J., Woolf, C. J., 2007. The neuropathic pain triad: neurons, immune cells and glia. Nat Neurosci 10, 1361-1368.

Scremin, O. U., Jenden, D. J., 1991. Time-dependent changes in cerebral choline and acetylcholine induced by transient global ischemia in rats. Stroke 22, 643-647.

Seguela, P., Wadiche, J., Dineley-Miller, K., Dani, J. A., Patrick, J. W., 1993. Molecular cloning, functional properties, and distribution of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium. J Neurosci 13, 596-604.

Sfikakis, P. P., 2010. The first decade of biologic TNF antagonists in clinical practice: lessons learned, unresolved issues and future directions. Curr Dir Autoimmun 11, 180-210.

Sharples, C. G., Wonnacott, S., 2001. Neuronal nicotinic receptors. Tocris reviews 19, 1-12.

Shen, J. X., Yakel, J. L., 2009. Nicotinic acetylcholine receptor-mediated calcium signaling in the nervous system. Acta Pharmacol Sin 30, 673-680.

Shu, X., Mendell, L. M., 1999. Nerve growth factor acutely sensitizes the response of adult rat sensory neurons to capsaicin. Neurosci Lett 274, 159-162.

Shubayev, V. I., Myers, R. R., 2000. Upregulation and interaction of TNFalpha and gelatinases A and B in painful peripheral nerve injury. Brain Res 855, 83-89.

Shytle, R. D., Mori, T., Townsend, K., Vendrame, M., Sun, N., Zeng, J., Ehrhart, J., Silver, A. A., Sanberg, P. R., Tan, J., 2004. Cholinergic modulation of microglial activation by alpha 7 nicotinic receptors. J Neurochem 89, 337-343.

Silverman, H. A., Dancho, M., Regnier-Golanov, A., Nasim, M., Ochani, M., Olofsson, P. S., Ahmed, M., Miller, E. J., Chavan, S. S., Golanov, E., Metz, C. N., Tracey, K. J., Pavlov, V. A., 2014. Brain region-specific alterations in the gene expression of cytokines, immune cell markers and cholinergic system components during peripheral endotoxin-induced inflammation. Mol Med 20, 601-611.

Skok, M. V., 2009. Editorial: To channel or not to channel? Functioning of nicotinic acetylcholine receptors in leukocytes. J Leukoc Biol 86, 1-3.

Soleimannejad, E., Naghdi, N., Semnanian, S., Fathollahi, Y., Kazemnejad, A., 2007. Antinociceptive effect of intra-hippocampal CA1 and dentate gyrus injection of MK801 and AP5 in the formalin test in adult male rats. Eur J Pharmacol 562, 39-46.

Sommer, C., Kress, M., 2004. Recent findings on how proinflammatory cytokines cause pain: peripheral mechanisms in inflammatory and neuropathic hyperalgesia. Neurosci Lett 361, 184-187.

Sommer, C., Lindenlaub, T., Teuteberg, P., Schafers, M., Hartung, T., Toyka, K. V., 2001. Anti-TNF-neutralizing antibodies reduce pain-related behavior in two different mouse models of painful mononeuropathy. Brain Res 913, 86-89.

Spengler, R. N., Sud, R., Knight, P. R., Ignatowski, T. A., 2007. Antinociception mediated by alpha(2)-adrenergic activation involves increasing tumor necrosis factor alpha (TNFalpha) expression and restoring TNFalpha and alpha(2)-adrenergic inhibition of norepinephrine release. Neuropharmacology 52, 576-589.

Stokes, C., Treinin, M., Papke, R. L., 2015. Looking below the surface of nicotinic acetylcholine receptors. Trends Pharmacol Sci 36, 514-523.

Sud, R., Spengler, R. N., Nader, N. D., Ignatowski, T. A., 2008. Antinociception occurs with a reversal in alpha 2-adrenoceptor regulation of TNF production by peripheral monocytes/macrophages from pro- to anti-inflammatory. Eur J Pharmacol 588, 217-231.

Sullivan, J. P., Decker, M. W., Brioni, J. D., Donnelly-Roberts, D., Anderson, D. J., Bannon, A. W., Kang, C. H., Adams, P., Piattoni-Kaplan, M., Buckley, M. J., et al., 1994. (+/-)-Epibatidine elicits a diversity of in vitro and in vivo effects mediated by nicotinic acetylcholine receptors. J Pharmacol Exp Ther 271, 624-631.

Sun, S. C., Ganchi, P. A., Ballard, D. W., Greene, W. C., 1993. NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. Science 259, 1912-1915.

Sung, K. W., Kirby, M., McDonald, M. P., Lovinger, D. M., Delpire, E., 2000. Abnormal GABAA receptor-mediated currents in dorsal root ganglion neurons isolated from Na-K-2Cl cotransporter null mice. J Neurosci 20, 7531-7538.

Suzuki, R., Morcuende, S., Webber, M., Hunt, S. P., Dickenson, A. H., 2002. Superficial NK1-expressing neurons control spinal excitability through activation of descending pathways. Nat Neurosci 5, 1319-1326.

Suzuki, T., Hide, I., Matsubara, A., Hama, C., Harada, K., Miyano, K., Andra, M., Matsubayashi, H., Sakai, N., Kohsaka, S., Inoue, K., Nakata, Y., 2006. Microglial alpha7 nicotinic acetylcholine receptors drive a phospholipase C/IP3 pathway and modulate the cell activation toward a neuroprotective role. J Neurosci Res 83, 1461-1470.

Svensson, C. I., Marsala, M., Westerlund, A., Calcutt, N. A., Campana, W. M., Freshwater, J. D., Catalano, R., Feng, Y., Protter, A. A., Scott, B., Yaksh, T. L., 2003. Activation of p38 mitogen-activated protein kinase in spinal microglia is a critical link in inflammation-induced spinal pain processing. J Neurochem 86, 1534-1544.

Sweitzer, S., De Leo, J., 2011. Propentofylline: glial modulation, neuroprotection, and alleviation of chronic pain. Handb Exp Pharmacol, 235-250.

Sweitzer, S. M., Pahl, J. L., DeLeo, J. A., 2006. Propentofylline attenuates vincristine-induced peripheral neuropathy in the rat. Neurosci Lett 400, 258-261.

Sweitzer, S. M., Schubert, P., DeLeo, J. A., 2001. Propentofylline, a glial modulating agent, exhibits antiallodynic properties in a rat model of neuropathic pain. J Pharmacol Exp Ther 297, 1210-1217.

Taly, A., Corringer, P. J., Guedin, D., Lestage, P., Changeux, J. P., 2009. Nicotinic receptors: allosteric transitions and therapeutic targets in the nervous system. Nat Rev Drug Discov 8, 733-750.

Tartaglia, M. C., Narayanan, S., De Stefano, N., Arnaoutelis, R., Antel, S. B., Francis, S. J., Santos, A. C., Lapierre, Y., Arnold, D. L., 2002. Choline is increased in pre-lesional normal appearing white matter in multiple sclerosis. J Neurol 249, 1382-1390.

Taylor, P. C., Feldmann, M., 2009. Anti-TNF biologic agents: still the therapy of choice for rheumatoid arthritis. Nat Rev Rheumatol 5, 578-582.

Tegeder, I., Niederberger, E., Schmidt, R., Kunz, S., Guhring, H., Ritzeler, O., Michaelis, M., Geisslinger, G., 2004. Specific Inhibition of IkappaB kinase reduces hyperalgesia in inflammatory and neuropathic pain models in rats. J Neurosci 24, 1637-1645.

Terada, M., Kuzumaki, N., Hareyama, N., Imai, S., Niikura, K., Narita, M., Yamazaki, M., Suzuki, T., Narita, M., 2008. Suppression of enriched environment-induced neurogenesis in a rodent model of neuropathic pain. Neurosci Lett 440, 314-318.

Thomsen, M. S., Mikkelsen, J. D., 2012. The alpha7 nicotinic acetylcholine receptor ligands methyllycaconitine, NS6740 and GTS-21 reduce lipopolysaccharide-induced TNF-alpha release from microglia. J Neuroimmunol 251, 65-72.

Timmermann, D. B., Gronlien, J. H., Kohlhaas, K. L., Nielsen, E. O., Dam, E., Jorgensen, T. D., Ahring, P. K., Peters, D., Holst, D., Christensen, J. K., Malysz, J., Briggs, C. A., Gopalakrishnan, M., Olsen, G. M., 2007. An allosteric modulator of the alpha7 nicotinic acetylcholine receptor possessing cognition-enhancing properties in vivo. J Pharmacol Exp Ther 323, 294-307.

Tomaz, V. S., Cordeiro, R. C., Costa, A. M., de Lucena, D. F., Nobre Junior, H. V., de Sousa, F. C., Vasconcelos, S. M., Vale, M. L., Quevedo, J., Macedo, D., 2014. Antidepressant-like effect of nitric oxide synthase inhibitors and sildenafil against lipopolysaccharide-induced depressive-like behavior in mice. Neuroscience 268, 236-246.

Tracey, K. J., 2009. Reflex control of immunity. Nat Rev Immunol 9, 418-428.

Trang, T., Beggs, S., Salter, M. W., 2011. Brain-derived neurotrophic factor from microglia: a molecular substrate for neuropathic pain. Neuron Glia Biol 7, 99-108.

Tsuda, M., Inoue, K., Salter, M. W., 2005. Neuropathic pain and spinal microglia: a big problem from molecules in "small" glia. Trends Neurosci 28, 101-107.

Tsuda, M., Shigemoto-Mogami, Y., Koizumi, S., Mizokoshi, A., Kohsaka, S., Salter, M. W., Inoue, K., 2003. P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. Nature 424, 778-783.

Turek, J. W., Kang, C. H., Campbell, J. E., Arneric, S. P., Sullivan, J. P., 1995. A sensitive technique for the detection of the alpha 7 neuronal nicotinic acetylcholine receptor antagonist, methyllycaconitine, in rat plasma and brain. J Neurosci Methods 61, 113-118.

Uesugi, M., Nakajima, K., Tohyama, Y., Kohsaka, S., Kurihara, T., 2006. Nonparticipation of nuclear factor kappa B (NFkappaB) in the signaling cascade of c-Jun N-terminal kinase (JNK)- and p38 mitogen-activated protein kinase (p38MAPK)-dependent tumor necrosis factor alpha (TNFalpha) induction in lipopolysaccharide (LPS)-stimulated microglia. Brain Res 1073-1074, 48-59.

Umana, I. C., Daniele, C. A., McGehee, D. S., 2013. Neuronal nicotinic receptors as analgesic targets: it's a winding road. Biochem Pharmacol 86, 1208-1214.

v Eitzen, U., Egensperger, R., Kosel, S., Grasbon-Frodl, E. M., Imai, Y., Bise, K., Kohsaka, S., Mehraein, P., Graeber, M. B., 1998. Microglia and the development of spongiform change in Creutzfeldt-Jakob disease. J Neuropathol Exp Neurol 57, 246-256.

Valencia-de Ita, S., Lawand, N. B., Lin, Q., Castaneda-Hernandez, G., Willis, W. D., 2006. Role of the Na+-K+-2Cl- cotransporter in the development of capsaicin-induced neurogenic inflammation. J Neurophysiol 95, 3553-3561.

van Westerloo, D. J., Giebelen, I. A., Florquin, S., Bruno, M. J., Larosa, G. J., Ulloa, L., Tracey, K. J., van der Poll, T., 2006. The vagus nerve and nicotinic receptors modulate experimental pancreatitis severity in mice. Gastroenterology 130, 1822-1830.

Vincler, M., 2005. Neuronal nicotinic receptors as targets for novel analgesics. Expert Opin Investig Drugs 14, 1191-1198.

Vincler, M., Wittenauer, S., Parker, R., Ellison, M., Olivera, B. M., McIntosh, J. M., 2006. Molecular mechanism for analgesia involving specific antagonism of alpha9alpha10 nicotinic acetylcholine receptors. Proc Natl Acad Sci U S A 103, 17880-17884.

Viviani, B., Bartesaghi, S., Gardoni, F., Vezzani, A., Behrens, M. M., Bartfai, T., Binaglia, M., Corsini, E., Di Luca, M., Galli, C. L., Marinovich, M., 2003. Interleukin-1beta enhances NMDA receptor-mediated intracellular calcium increase through activation of the Src family of kinases. J Neurosci 23, 8692-8700.

Walter, D. S., Flockhart, I. R., Haynes, M. J., Howlett, D. R., Lane, A. C., Burton, R., Johnson, J., Dettmar, P. W., 1984. Effects of idazoxan on catecholamine systems in rat brain. Biochem Pharmacol 33, 2553-2557.

Wang, H., Liao, H., Ochani, M., Justiniani, M., Lin, X., Yang, L., Al-Abed, Y., Wang, H., Metz, C., Miller, E. J., Tracey, K. J., Ulloa, L., 2004. Cholinergic agonists inhibit HMGB1 release and improve survival in experimental sepsis. Nat Med 10, 1216-1221.

Wang, H., Yu, M., Ochani, M., Amella, C. A., Tanovic, M., Susarla, S., Li, J. H., Wang, H., Yang, H., Ulloa, L., Al-Abed, Y., Czura, C. J., Tracey, K. J., 2003. Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. Nature 421, 384-388.

Wang, Y., Su, D. M., Wang, R. H., Liu, Y., Wang, H., 2005. Antinociceptive effects of choline against acute and inflammatory pain. Neuroscience 132, 49-56.

Ward, A., Heel, R. C., 1984. Bumetanide. A review of its pharmacodynamic and pharmacokinetic properties and therapeutic use. Drugs 28, 426-464.

Watkins, L. R., Hutchinson, M. R., Ledeboer, A., Wieseler-Frank, J., Milligan, E. D., Maier, S. F., 2007. Norman Cousins Lecture. Glia as the "bad guys": implications for improving clinical pain control and the clinical utility of opioids. Brain Behav Immun 21, 131-146.

Watkins, L. R., Maier, S. F., 2003. Glia: a novel drug discovery target for clinical pain. Nat Rev Drug Discov 2, 973-985.

Watkins, L. R., Milligan, E. D., Maier, S. F., 2001. Glial activation: a driving force for pathological pain. Trends Neurosci 24, 450-455.

Watkins, L. R., Nguyen, K. T., Lee, J. E., Maier, S. F., 1999. Dynamic regulation of proinflammatory cytokines. Adv Exp Med Biol, pp. 153-178.

Wei, X. H., Zang, Y., Wu, C. Y., Xu, J. T., Xin, W. J., Liu, X. G., 2007. Peri-sciatic administration of recombinant rat TNF-alpha induces mechanical allodynia via upregulation of TNF-alpha in dorsal root ganglia and in spinal dorsal horn: the role of NF-kappa B pathway. Exp Neurol 205, 471-484.

Wen, Y. R., Tan, P. H., Cheng, J. K., Liu, Y. C., Ji, R. R., 2011. Microglia: a promising target for treating neuropathic and postoperative pain, and morphine tolerance. J Formos Med Assoc 110, 487-494.

Williams, D. K., Peng, C., Kimbrell, M. R., Papke, R. L., 2012. Intrinsically low open probability of alpha7 nicotinic acetylcholine receptors can be overcome by positive

allosteric modulation and serum factors leading to the generation of excitotoxic currents at physiological temperatures. Mol Pharmacol 82, 746-759.

Williams, D. K., Wang, J., Papke, R. L., 2011. Positive allosteric modulators as an approach to nicotinic acetylcholine receptor-targeted therapeutics: advantages and limitations. Biochem Pharmacol 82, 915-930.

Williams, M., Sullivan, J., Arneric, S., 1994. Neuronal nicotinic acetylcholine receptors. Drug News Perspect 7, 205-205.

Wilms, H., Rosenstiel, P., Sievers, J., Deuschl, G., Zecca, L., Lucius, R., 2003. Activation of microglia by human neuromelanin is NF-kappaB dependent and involves p38 mitogen-activated protein kinase: implications for Parkinson's disease. FASEB J 17, 500-502.

Wongtrakool, C., Roser-Page, S., Rivera, H. N., Roman, J., 2007. Nicotine alters lung branching morphogenesis through the alpha7 nicotinic acetylcholine receptor. Am J Physiol Lung Cell Mol Physiol 293, L611-L618.

Woolf, C. J., Salter, M. W., 2000. Neuronal plasticity: increasing the gain in pain. Science 288, 1765-1769.

Wu, S., Zhao, H., Luo, H., Xiao, X., Zhang, H., Li, T., Zuo, X., 2014. GTS-21, an alpha7-nicotinic acetylcholine receptor agonist, modulates Th1 differentiation in CD4+ T cells from patients with rheumatoid arthritis. Exp Ther Med 8, 557-562.

Xie, W., Liu, X., Xuan, H., Luo, S., Zhao, X., Zhou, Z., Xu, J., 2006. Effect of betamethasone on neuropathic pain and cerebral expression of NF-kappaB and cytokines. Neurosci Lett 393, 255-259.

Xu, Q., Yaksh, T. L., 2011. A brief comparison of the pathophysiology of inflammatory versus neuropathic pain. Curr Opin Anaesthesiol 24, 400-407.

Yajima, Y., Narita, M., Narita, M., Matsumoto, N., Suzuki, T., 2002. Involvement of a spinal brain-derived neurotrophic factor/full-length TrkB pathway in the development of nerve injury-induced thermal hyperalgesia in mice. Brain Res 958, 338-346.

Yaksh, T. L., 1985. Pharmacology of spinal adrenergic systems which modulate spinal nociceptive processing. Pharmacol Biochem Behav 22, 845-858.

Yoon, S. Y., Patel, D., Dougherty, P. M., 2012. Minocycline blocks lipopolysaccharide induced hyperalgesia by suppression of microglia but not astrocytes. Neuroscience 221, 214-224.

Yoshikawa, H., Kurokawa, M., Ozaki, N., Nara, K., Atou, K., Takada, E., Kamochi, H., Suzuki, N., 2006. Nicotine inhibits the production of proinflammatory mediators in human monocytes by suppression of I-kappaB phosphorylation and nuclear factor-kappaB transcriptional activity through nicotinic acetylcholine receptor alpha7. Clin Exp Immunol 146, 116-123.

Yue, Y., Liu, R., Cheng, W., Hu, Y., Li, J., Pan, X., Peng, J., Zhang, P., 2015. GTS-21 attenuates lipopolysaccharide-induced inflammatory cytokine production in vitro by modulating the Akt and NF-kappaB signaling pathway through the alpha7 nicotinic acetylcholine receptor. Int Immunopharmacol 29, 504-512.

Zhang, J. C., Wu, J., Fujita, Y., Yao, W., Ren, Q., Yang, C., Li, S. X., Shirayama, Y., Hashimoto, K., 2015. Antidepressant effects of TrkB ligands on depression-like behavior and dendritic changes in mice after inflammation. Int J Neuropsychopharmacol 18.

Zhang, W., Liu, L. Y., Xu, T. L., 2008. Reduced potassium-chloride co-transporter expression in spinal cord dorsal horn neurons contributes to inflammatory pain hypersensitivity in rats. Neuroscience 152, 502-510.

Zhao, J., Seereeram, A., Nassar, M. A., Levato, A., Pezet, S., Hathaway, G., Morenilla-Palao, C., Stirling, C., Fitzgerald, M., McMahon, S. B., Rios, M., Wood, J. N., 2006. Nociceptor-derived brain-derived neurotrophic factor regulates acute and inflammatory but not neuropathic pain. Mol Cell Neurosci 31, 539-548.

Zhou, X. F., Deng, Y. S., Xian, C. J., Zhong, J. H., 2000. Neurotrophins from dorsal root ganglia trigger allodynia after spinal nerve injury in rats. Eur J Neurosci 12, 100-105.

Zhuo, M., Wu, G., Wu, L. J., 2011. Neuronal and microglial mechanisms of neuropathic pain. Mol Brain 4, 31.

Zielasek, J., Hartung, H. P., 1996. Molecular mechanisms of microglial activation. Adv Neuroimmunol 6, 191-122.

Zimmerman, M. E., Pan, J. W., Hetherington, H. P., Lipton, M. L., Baigi, K., Lipton, R. B., 2009. Hippocampal correlates of pain in healthy elderly adults: a pilot study. Neurology 73, 1567-1570.