ROLE OF *N*-METHYL-*D*-ASPARTATE RECEPTORS IN THE REGULATION OF HUMAN AIRWAY SMOOTH MUSCLE FUNCTION AND AIRWAY RESPONSIVENESS

BY

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ABSTRACT

Increased airway smooth muscle (ASM) mass contributes to airway hyperresponsiveness (AHR) in asthma and is orchestrated by growth factors, cytokines and chemokines. Airway contractile responses are influenced by neuromediators, such as acetylcholine, and glutamate released by parasympathetic and sympathetic airway nerves. Hyperactivity of these neural elements, termed neurogenic inflammation, is linked with hypercontractility and AHR. Glutamate is a non-essential amino acid derivative, and its physiological role is traditionally considered with respect to its being the primary excitatory neurotransmitter in brain, and regulation of neuronal development and memory. In allergic inflammation, immune cells including dendritic cells, neutrophils and eosinophils, constitutively synthesize and release glutamate, which signals through activation of glutamate receptors, most important among which are ionotrophic *N*-methyl *D*-aspartate receptors (NMDA-R). We hypothesized that glutamatergic signaling mediated through NMDA-Rs plays an important role in inducing functional Ca^{2+} responses in human (H) ASM cells that can underpin airway hypercontractility. We investigated the expression of functional NMDA-Rs in HASM cells, and assessed the effects of pro-inflammatory cytokines on NMDA-R expression and functional responses. Moreover, we measured airway responses to NMDA in mice, murine thin cut lung slice preparations, and floating collagen gels seeded with HASMs. Our data reveal that airway myocytes express multi-subunit NMDA-R complexes that function as receptor-operated calcium channels (ROCCs), mobilizing intracellular Ca^{2+} in ASM *in vitro* and airway contraction *ex vivo*. Individual airway myocytes treated with NMDA-R agonist exhibit disparate temporal patterns of intercellular Ca^{2+} flux that can be partitioned into four discrete function sub-groups. Further we show that tumor

necrosis factor (TNF) exposure modulates NMDA-R subunit expression, and these changes are associated with a shift in the distribution of myocytes in individual Ca^{2+}-mobilization sub-groups *in vitro*. Further, post-TNF exposure, NMDA-R agonists' treatment induced Ca^{2+}-dependent airway *dilation* in murine lung slice preparations, an effect that was prevented by co-treatment with inhibitors of nitric oxide synthase (NOS) or cyclooxygenase (COX). Taken together, we conclude that NMDA-R regulate HASM-mediated airway contraction and their role can be affected upon exposure to asthma-associated inflammatory mediators. Thus, NMDA-Rs are of relevance to mechanisms that determine airway narrowing and AHR associated with chronic respiratory diseases.

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DEDICATION

To myself, for enduring all the hassles and displaying tremendous resilience during adversity

To my lovely wife "Aruna", who has always been a source of inspiration and pillar of support ever since she came into my life....always cherish your company

Dedicated to my beautiful daughter "Nandini", who always been a source of joy and delight to my family ever since she was born....love you princess

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&

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
ADAR2	Adenosine deaminase acting on RNA2
AHR	Airway hyperresponsiveness
ALI	Acute lung injury
ANOVA	Analysis of variance
AMPA-Rs	a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
AP-1	activator protein 1
ARC	Arachidonic-acid regulated
ASM	Airway smooth muscle
AR	Airway remodeling
BAL	Bronchoalveolar lavage
BDNF	Brain-derived neurotrophic factor
β2AR	beta-adrenergic receptor
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
BT	Bronchial thermoplasty
Ca^{2+}	Calcium ions
$[Ca^{2+}]_i$	Intracellular calcium
c – ADPR	Cyclic adenosine diphosphate ribose
cAMP	Cyclic adenosine monophosphate
CAM	Calmodulin
c/EBP – α	CCAAT/enhancer binding protein - α
CDKs	Cyclin-dependent kinases
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CGRP	Calcitonin gene-related peptide
CICR	Calcium-induced calcium release

CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CSB	Cytoskeleton buffer
CTGF	Connective tissue growth factor
CTLA-4	Cytotoxic T-lymphocyte antigen – 4
CPI – 17	C-kinase-potentiated-phosphatase inhibitor 17
CysLTs	Cysteinyl leukotrienes
D-AP5	D-(-)-2-Amino-5-phosphonopentanoic acid
DAG	1, 2 – diacyl glycerol
DCs	Dendritic cells
DI	Deep inspirations
DMEM	Dulbecco's modified eagle's medium
E2F	Elongation factor 2
EDTA	Ethylene diaminetetraacetic acid
ECM	Extracellular matrix
ECP	Eosinophil cationic protein
EDN	Eosinophil-derived neurotoxin
EDTA	Ethylene diaminetetracetic acid
EMT	Epithelial-mesenchymal transition
EPSP	Excitatory postsynaptic potential
ERK	extracellular signal-regulated kinase
FBS	Fetal bovine serum
FEV1	Forced expiratory volume in 1 second
FGF10	Fibroblast growth factor 10
G	Airway resistance
GABA	Gamma-amino butyric acid
GEFs	Guanine nucleotide exchange factors

GOLD	Global initiative on Obstructive Lung Disease
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPCR	G-protein coupled receptor
GTPase	Guanosine triphosphotase
H	Tissue elastance
HASM	Human airway smooth muscle cells
HDACs	Histone deacetylases
HDM	House dust mite
HBSS	Hanks buffered saline solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHB	HBSS/HEPES buffer containing 0.1% BSA
hTERT	Human telomerase reverse transcriptase
ICS	Inhaled corticosteroids
IFN-γ	Interferon gamma
IDO	indoleamine 2, 3-dioxygenase
iGluRs	ionotrophic glutamate receptors
IL	Interleukin
IL-4	Interleukin-4
IL-1β	Interleukin-1β
ISO	Isoproterenol
ITS	Insulin-transferrin-selenium
JNK	c-Jun-N-terminal kinase
K-H	Krebs – Henseleit bicarbonate solution
KLF4	Kruppel like factor 4
KYN	Kynurenic acid
LABA	Long acting beta agonists
L-NAME	L-N^G-nitroarginine methyl ester
LTD	Long term depression

LTP	Long term potentiation
MAGUKs	Membrane – associated guanylate kinases
MCh	Methacholine
MC	Mast cells
MCP	Macrophage chemoattractant protein
MEK1	MAPK/ERK kinase 1
mGluRs	Metabotrophic glutamate receptors
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MK-801	Dizocilpine
MLC20	Myosin light chain phosphorylation
MLCP	Myosin light chain phosphotase
Mφ	Macrophages
MMP	matrix metalloproteinase
MSC	Mesenchymal stem cells
MYPT1	Myosin phosphotase 1
NANC	Non-adrenergic non-cholinergic
NCX	Sodium calcium exchanger
NF-AT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
NKA	Neurokinin A
NMDA	*N*-methyl-*D*-aspartate
NMDA-R	*N*-methyl-*D*-aspartate receptor
NO	Nitric oxide
NOS	Nitric oxide synthase
NT	Neurotrophin
OX40L	OX-40 ligand

PAF	Platelet-activating factor
PAK1	p21-activated kinase 1
PBS	Phosphate buffered saline
PCP	Phencyclidine
PDGF	Platelet-derived growth factor
PDK1	Phosphoinositide-dependent kinase 1
PEEP	Positive end expiratory pressure
PFA	Paraformaldehyde
PI3-K	Phosphoinositide-3 kinase
PIP2	Phosphatidylinositol 4, 5 – bisphosphate
PLC-β	Phospholipase –C β
PM	Plasma membrane
PMN	Polymorphonuclear
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKC-ε	Protein kinase C epsilon
QUIN	Quinolinic acid
R_{aw}	Airway resistance
Rb	Retinoblastoma protein
RhoA	Ras homolog gene family member A
ROCE	Receptor operated calcium entry
ROCC	Receptor operated calcium channels
ROK/ROCK	Rho-associated protein kinase
RTK	Tyrosine kinase
RYRs	Ryanodine receptors
SEM	Standard error of mean
SERCA2	Sarcoendoplasmic reticulum Ca2+ ATPase 2

SMA	α-smooth muscle actin
SHH	Sonic hedgehog
smMHC	smooth muscle myosin heavy chain
smMLC	smooth muscle myosin light chain
smMLCK	smooth muscle myosin light chain kinase
SOCE	Store operated calcium entry
SOCC	Store operated calcium channels
SR	Sarcoplasmic reticulum
SRF	Serum response factor
STIM1	Stromal-interacting molecule 1
TBST	Tris-buffered saline containing Tween-20
TCLS	Thin cut lung slice
TLR	Toll-like receptor
TNF	Tumor necrosis factor-alpha
TGF-β	Transforming growth factor beta
Th	T-helper cell
Th1	T-helper cell type-1
Th2	T-helper cell type-2
TSLP	Thymic stromal lymphopoietin
Treg	Regulatory T cell
TRP	Transient receptor potential
TRPC	Transient receptor potential C
VIP	Vasoactive intestinal peptide
Vmax	Maximum shortening velocity
WNT	Wingless integrase

1.0 CHAPTER 1

INTRODUCTION

1.1.0 AIRWAY SMOOTH MUSCLE

Present in the respiratory airways of vertebrates, airway smooth muscle (ASM) compartment was first described in 1822 by F.D Reissessen [1] as a specialized contractile apparatus of the lung that geodesically encircles airways. Located prominently in the central (referring to cartilaginous or segmental bronchi with an internal diameter ranging between 1.3mm and 11mm) and peripheral airways (referring to non-cartilaginous respiratory bronchioles with an internal diameter between 0.3mm – 1.3mm) [1], ASM cells are packed in the form of bundles or fascicles. These bundles provide mechanical support to airways and regulates airway lumen diameter owing to its contractile ability. Considered to be an important determinant of lung and airway physiology in health and disease, ASM bundles also facilitate mucus clearance and regulate bronchomotor tone through internal Ca^{2+} oscillations [1, 2]. Additionally, ASM is involved in numerous signaling pathways associated with lung development, injury, repair and inflammation, altogether emphasizing its role as a pleiotropic tissue [2, 3].

1.1.1 ASM in health

Originally, human ASM tissue was referred by some as an “appendix of the lung” [4], which means a rudimentary structure without any appreciable physiological advantage. However, current research on ASM biology in the past 2 decades reveal a progressive shift in opinion towards ASM as an “architect of the lung” [5].

The development, origin and differentiation of airway myocytes are still debatable. Early investigations identified that ASM cells originate prior to vascular smooth muscle that coincides with the formation of lung bud [5, 6]. Lung mesenchymal progenitors, which appear at the beginning of pseudoglandular phase of lung formation (gestation day 11 in mouse and week 5 in humans) are believed to be responsible in the formation of smooth muscle lining along a developing bronchial tree in a proximal-to-distal pattern as the lung enlarges [5-7]. In particular, distal lung mesenchymal cells expressing fibroblast growth factor (FGF10) differentiate into bronchial smooth muscle cells [8]. These studies used sequential expression and distribution of contractile and cytoskeletal proteins (*e.g.*, smooth muscle α-actin, SM-22, smooth muscle myosin heavy chain isoforms SM-1, SM-2 and SM-B) during differentiation as an identification marker [6, 8]. ASM development is also regulated by intracellular signaling networks (*e.g.*, RhoA) and epithelium-derived morphogenic factors (*e.g.*, transforming growth factor β (TGF-β), platelet-derived growth factor (PDGF), sonic hedgehog (SHH), retinoic acid and bone morphogenetic protein -4 (BMP-4)). Of critical importance in ASM origin and differentiation are mechanical factors such as degree of mechanical strain from the parenchyma, changes in intraluminal hydrostatic bronchial pressure, and the ability of mesenchymal cells to elongate/stretch or change shape over a defined surface [5, 8].

Airway myogenesis during lung development can be broadly categorized into two phases: pre-natal and post-natal [9]. During pre-natal phase, airway myocytes undergo an organized pattern of development to form a uniformly continuous meshwork of interconnected muscle fibers [9]. These bundles exhibit a rhythmic and spontaneous

contractile behavior during early fetal life and are termed as “phasic ASM bundles”. In post-natal stage of development, ASM bundles are re-organized perpendicularly into compact networks under the influence of stress-induced cellular signals [9]. Such an arrangement is presumed to be essential for airways to maintain tone, become stiffer and exhibit increased strength, thereby preventing them from collapsing during bronchial contraction [9]. In addition, distinct organization of ASM bundles even prevents airway resistance during expiration, thereby allowing a normalized tidal breathing cycle [9].

Cellular heterogeneity and capacity, *in vitro* and *in vivo*, for phenotypic and mechanical plasticity are features of ASM bundles [10]. Spontaneous switching of mature myocytes between proliferative, contractile, migratory and synthetic phenotypes provides them the capability to regulate myriad cellular processes including synthesis of extracellular matrix (ECM) proteins and release of various secretory molecules including cytokines, growth factors and chemokines [11]. However, the unique ability of airway myocytes to undergo abnormal structural and functional transformation (as outlined in the next section) when exposed to allergens, inhaled environmental pollutants, microbes, viruses and other inflammatory mediators renders this multifaceted tissue an attractive therapeutic target for chronic respiratory diseases.

1.1.2 ASM in Disease: Asthma, COPD and Cystic Fibrosis

Earliest observations on ASM’s role in respiratory diseases were by Sir Henry Hyde Salter as early as in 1860, when he defined asthma as “paroxysmal dyspnoea of a peculiar

character with intervals of healthy respiration between attacks", possibly due to abnormal airway narrowing [12, 13]. Henry acknowledged even then that ASM undergoes remodeling (thickening) and contributes to airflow limitation, and declining lung function. This contribution to pathophysiology is also evident in other chronic inflammatory diseases of conducting airways including chronic obstructive pulmonary disorder (COPD) [14] and cystic fibrosis [15].

Asthma: Asthma is a highly heterogeneous chronic inflammatory condition of the airways that exists in various forms with discrete pathological mechanisms [16, 17]. Currently, around $56 billion is spent by US annually towards direct and indirect cost of asthma management, signifying its disproportionately high economic burden on the health care system [18]. According to the Canadian Asthma Consensus, asthma is described as *"paroxysmal or persistent symptoms, such as dyspnoea, chest tightness, wheezing, sputum production and cough associated with variable airflow limitation and airway hyperresponsiveness to endogenous or exogenous stimuli. Inflammation and its resultant effects on airway structure are considered the main mechanisms leading to the development and persistence of asthma"* [19]. The current recommended treatment for asthma is a combination of inhaled corticosteroids (ICS) like budesonide and long-acting β-agonists (LABAs) like formoterol [20]. However, severe asthmatics refractory to corticosteroids require additional therapeutic interventions for disease management.

Elegant histological examinations done by Huber and Koessler [21] on airway tissues from asthmatics demonstrated marked increase in muscle thickness around airways due to asthma [21], thereby acknowledging ASM's contribution in the development of airway hyperresponsiveness (AHR) and airway remodeling (AR) considered to be the cardinal features of asthma. Dysfunctional airway myocytes contribute to maintenance of inflammatory milieu in and around the airway lumen causing acute exacerbations, reversible bronchial obstruction, excessive mucus production, sub-epithelial fibrosis and extreme damage to the epithelial barrier [13, 22]. Also, persistent airway inflammation, contributed partly by the ability of ASM cells to secrete pro-inflammatory molecules in response to various stimuli [23], induces excessive proliferation of airway myocytes (ASM hyperplasia), migration and increased cell size (ASM hypertrophy), thereby causing augmented airway constriction, which emphasizes the importance of ASM compartment in asthma disease progression and pathophysiology [24].

COPD: As per the Global Initiative on Obstructive Lung Disease (GOLD), *"COPD is a disease characterized by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response in the lungs to noxious particles or gases" [25]*. Approximately ~5% of total world population [26], COPD pathophysiology is characterized by emphysema (breakdown of lung parenchyma) and obstructive bronchiolitis (non-specific chronic neutrophilic inflammatory state causing small airway obstruction) in genetically-susceptible individuals due to incessant exposure to tobacco smoke, indoor air pollutants, exhaust gases and urban air particulates [25-27]. Apart from diminished elastic recoiling

ability of lung, compromised lung compliance and destruction of alveolar compartments, COPD disease severity is correlated with distinct changes occurring in the structural tissues surrounding the airways including chronic mucus secretion, goblet cell hyperplasia, fibrosis in peribronchial spaces and sub-epithelial basement membrane, thickened epithelial barrier and submucosal gland hypertrophy [14, 27, 28]. Alterations in ASM layer is another most prominent change observed in COPD small airways. Increased smooth muscle thickness, both as a result of increased cell mass or number, correlates positively with disease severity and lung function, measured as per predicted $FEV_{1\%}$ [14, 28]. $FEV_{1\%}$ is defined as the ratio of forced expiratory volume in 1 second (FEV1) to forced vital capacity (FVC) of an individual during a spirometry test. Information on ASM's contribution to disease pathogenesis is limited. However, studies have shown that small airways from COPD patients generate superior isometric force and stress, which in turn corresponds to exaggerated non-specific airway constriction [29]. In addition, the ability of airway myocytes to induce TGF-β-mediated fibrosis, promote epithelial-mesenchymal transition (EMT), release of extracellular matrix proteins and their inability to respond optimally to β-agonists or corticosteroids warrants further investigation to obtain better treatment options [14].

Cystic Fibrosis (CF): Also known as mucoviscodosis, *CF* is a recessive genetic disorder that primarily affects lung due to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein, thereby causing dysfunctional CFTR ion-channel leading to abnormal transport of Na^+ and Cl^- ions across epithelial membranes [15, 30]. *CF* patients demonstrate pulmonary manifestations that resemble asthmatics, including

difficulty in breathing, wheezing, sputum production, cough, airflow obstruction and AHR [31]. Emerging evidence suggests altered ASM is a key effector causing increased AHR in *CF* as airway myocytes express CFTR channels that participate in bronchodilation [32, 33]. Interestingly, *Cftr*$^{-/-}$ null mice exhibit diminished pulmonary nerve innervations, decreased ASM mass, altered contractility and reduced airway tone [34]; while loss of CFTR channel causes abnormal tracheal development in young children [35]. Also, presence of increased ASM mass and airway remodeling in *CF* patients [15] signals to the observation that smooth muscle in the airways may have a strong association with CF pathogenesis.

1.1.3 ASM Microenvironment –Immune Cells and their mediators

Inability to achieve homeostasis between tissue injury and repair mechanisms is believed to be the crux underlying ASM remodeling in asthma and COPD. This imbalance is orchestrated perpetually by a complex interplay of innate and adaptive immune cells and their secreted multiple pro-inflammatory cytokines present in local microenvironment. Outlined below is an overview about these immune cells and cytokines affecting ASM function in health and disease (Figure 1.1.3)

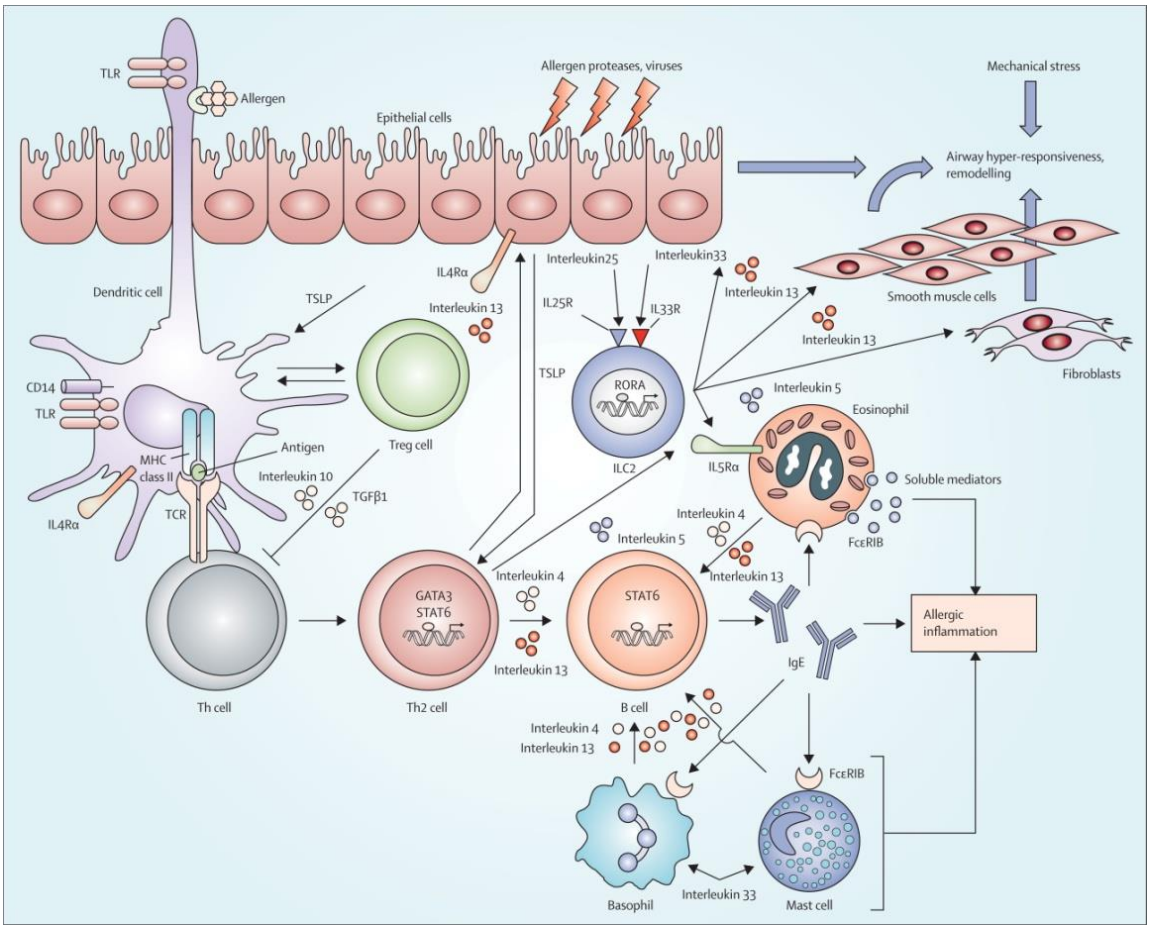

Figure 1.1.3 - Major immune pathways involved in asthma pathogenesis. Innate and addaptive components of allergic inflammation are shown, including the recently discovered ILC2 cells that release Th2 cytokines in response to epithelial damage. The links between immune responses and structural changes in the lung are also depicted. TLR=toll-like receptor. TSLP= thymic stromal lymphopoietin. TGFβ1=transforming growth-factor β1. TCR=T-cell receptor. Treg=regulatory T cell. Th=T-helper cell. Th2=T-helper-type-2 cell. (Adapted from Martinez *et al*, The Lancet, Volume 382, Issue 9901, 2013, 1360 – 1372)

Immune cells *(including eosinophils, neutrophils, basophils, mast cells, lymphocytes and dendritic cells)* are critically involved in propagating inflammatory airway responses, regulating allergic reactions and orchestrating ASM pathogenesis [36-40]. Involvement of eosinophils, neutrophils and T lymphocytes in exacerbating AHR and

AR responses during asthma is well-documented; their numbers are significantly elevated in the bronchoalveolar lavage fluid (BALF), sputum and airway specimens from asthmatics and correlate with disease severity [41-46]. Also, tissue biopsies from severe asthmatics and chronic smokers demonstrate a substantial infiltration of MCs into ASM tissue [47, 48]. DCs function as professional allergen-presenting cells to T and B-lymphocytes and initiate tolerogenic immune responses to allergen via activation of regulatory T cells, thereby acting as master regulators of adaptive immune responses [49]. During airway inflammation, activated granulocytes exhibit a unique pattern of cell surface receptors and secrete a wide-array of *de novo* synthesized granule-stored mediators and produce reactive oxygen molecules. Additionally, these cells along with lymphocytes release a number of pro-inflammatory mediators that can cause significant tissue injury, epithelial damage, fibrosis, ECM expansion, collagen deposition, angiogenesis, epithelial-mesenchymal transition, goblet cell metaplasia, leukocyte infiltration, vascular permeability, edema and immune dysfunction [38, 44, 45, 50, 51]. Further, *in vivo* localization of immune cells within the ASM bundles enhances smooth muscle contractility to agonists, and promotes ASM differentiation [41, 42, 52-54].

As a part of allergen clearance and tissue repair, inflammatory and structural cells also release various inflammatory mediators into the airway milieu, including pro-inflammatory cytokines (such as tumor necrosis factor (TNF), interleukin 4 (IL-4), interleukin 13 (IL-13), interleukin 5 (IL-5), thymic stromal lymphopoietin (TSLP) and transforming growth factor beta (TGF-β)), chemokines and growth factors that affect ASM phenotype and function in an autocrine/paracrine manner. These mediators, both alone or

in combination, contributes to ASM hyperplasia, promotes proliferative and synthetic phenotypes in ASM cells, impacts turnover rate of ECM proteins in cultured HASM cells [55-57], promotes ASM migration and glucocorticoid-insensitivity and induces ASM hypercontractility to various contractile agonists (such as carbachol, bradykinin, acetylcholine and histamine) via modifying intracellular Ca^{2+} sequestration in human ASM cells [22, 58-65]. Further research is required to delineate their individual contributions and the possibility of becoming important therapeutic targets for airway diseases.

1.1.4 Airway Responsiveness and ASM

Airway hyperresponsiveness (AHR), also termed as bronchial hyperresponsiveness, is characterized by excessive airway lumen narrowing in response to non-specific stimuli that causes considerable airflow limitation and can underpin dyspnea, a cardinal feature of asthma. Landmark investigations done by Lambert *et al* point to the fact that a dysfunctional ASM contraction is central to disoriented airway responses [66]. The primary role of the smooth muscle layer encircling conducting airways is to contract and relax, thereby allowing bi-directional airflow, achieve optimal pulmonary inflation and facilitate adequate gas exchange [67]. ASM is essential for maintaining bronchial tone, stabilizing airways during extreme changes in transmural pulmonary pressure caused during exercise or other excited states, and facilitating cough, a form of forced expiration for expulsion of germs and foreign material from lungs [67]. In obstructive airway disorders like asthma, the ASM layer is believed to be intrinsically dissimilar and contributes to AHR in multiple ways including (i) increased ASM mass (ii) increased SM

shortening velocity and force (iii) inadequate airway relaxation and (iv) inability to respond to deep inspirations [67, 68]. Analysis of bronchial biopsy samples from asthmatics indicate increased smooth muscle area, either due to cellular hyperplasia or hypertrophy, correlates positively with asthma severity and duration of disease [68]. Stimuli that promote ASM proliferation include TGFβ1, PDGF, EGF and IgE, which activate receptor tyrosine kinases and induce mitogenesis in a phosphoinositide-3 kinase (PI-3K) and p42/44 extracellular signal-regulated kinase (ERK) manner. Secreted by both infiltrating immune cells and resident structural cells including ASM cells, levels of these mediators are profoundly increased during chronic inflammation [69]. Pathological significance of increased ASM mass is further substantiated from clinical studies for bronchial thermoplasty (BT), a FDA-approved novel non-invasive and non-pharmacological therapeutic intervention for patients suffering with chronically severe asthma [70]. The procedure involves selective delivery of high-energy radiofrequency waves to remove ASM mass [70]. Data from randomized clinical trials suggests BT significantly improves patient quality of life together with drastic reduction in bronchial exacerbations, stable lung function, and decreased use of asthma medications [70]. Altogether, increased ASM mass can be considered as an important indicator of airway pathogenesis; albeit observations indicate myocyte proliferation is not the only contributing factor to the process [69]. Alternatively, migration of fibrocytes, pericytes and mesenchymal stem cells (MSCs) can underpin ASM hyperplasia, as well as suppressed ASM apoptosis [71]. The presence of increased muscle mass around airways is proposed to generate a considerably greater force, which causes hypercontractility.

The contractile apparatus in ASM cells is primarily composed of F-actin and myosin protein filaments, whose interactions and formation of actin-myosin crossbridges is controlled by multiple regulatory kinases and phosphatases. While F-actin filaments are associated with small contractile proteins such as caldesmon, tropomyosin and calponin, myosin filaments are primarily made up of a smooth muscle myosin heavy chain (smMHC) that binds to 2 myosin light chains (smMLC). In ASM cells, (i) force-generating capacity and (ii) shortening velocity (Vmax) of ASM cells regulates their ability to contract [72]. These parameters are in turn regulated by cytoskeletal arrangement of contractile filaments and actin-myosin crossbridges. For instance, a parallel assembly of these bridges generates greater force [72]. On the contrary, a serial arrangement of myosin-actin filaments causes increased Vmax, which causes greater myosin light chain phosphorylation (MLC_{20}) catalyzed by calmodulin-sensitive smooth muscle myosin light chain kinase (smMLCK) [72]. MLC20 phosphorylation initiates crossbridge formation through actin-myosin cycling leading to ASM shortening and contraction [73]. MLC_{20} dephosphorylation, regulated by myosin light chain phosphotase (MLCP) terminates the cycling process and causes ASM relaxation [73]. This process is partly regulated due to augmentation in intracellular Ca^{2+} levels in a RhoA/ROCK-dependent manner [74], a process known as excitation-contraction (E-C) coupling that is elaborately discussed in the next section. It is widely postulated that ASM cells from asthmatics and COPD patients demonstrate excessive smMLCK expression [74, 75], which correlates to significant changes in E-C coupling. In addition, the presence of inflammatory mediators in the milieu causes substantial increase in the expression of other contractile apparatus proteins including

caldesmon, alpha-smooth muscle actin (α-SMA), calponin and SM22, which promotes greater airway contractility [75].

In normal individuals, a bronchodilatory role for deep inspirations (DI), thereby maximally opening airways is well-recognized [76]. However, airways from asthma and COPD patients fail to relax and open adequately after a DI [76, 77]. In healthy individuals, DI avoidance leads to transient AHR to methacholine that is similar to those observed in asthmatics [78]. One possible explanation for this phenomenon can be activation of stretch receptors on ASM cells causing release of myocyte relaxants such as nitric oxide (NO) [67].

Currently, LABAs are the most-established ASM relaxants; they bind to β-adrenergic receptors (β2AR), which leads to opening of K^+ ion channels, cAMP generation and activation of protein kinase A (PKA) causing relaxation of ASM cells. Albeit, frequent exposure to β-agonists causes β2AR internalization and desensitization on ASM cells, thereby causing diminished β-agonist responsiveness [69]. The presence of proinflammatory Th2 cytokines, particularly IL-1β and IL-13, further potentiates this occurrence [69]. ASM-mediated airway responsiveness is also influenced by excitatory cholinergic parasympathetic neural networks innervating ASM bundles [73]. Cholinergic responses are mediated by acetylcholine, an excitatory neurotransmitter that functions through activation of muscarinic receptors, particularly of M3 subtype expressed on ASM cells. Additionally, emerging evidence suggests ASM tone is regulated by non-adrenergic-

non-cholinergic (NANC) neuromediators, which include substance P, vasoactive intestinal peptide (VIP), nerve growth factor (NGF), neurokinins and glutamate [73, 79, 80]. However, further investigations are warranted to delineate the role of these molecules. Because of their heterogeneous cellular origin and function, airway myocytes are central mediators of AR and modulators of local inflammatory responses, other hallmark features of asthma. Cellular heterogeneity of ASM cells has been discussed in the subsequent sections.

1.1.5 Proliferative, Synthetic and Contractile Phenotypes of ASM

Structural changes of the airways are a hallmark feature of chronic lung diseases, including asthma. In 1922, Huber and Koessler [21] first described airway remodeling (AR) as a complex multicellular process caused by serious organizational and compositional rearrangement in the structural components around airways that culminates as airway wall thickening, airway narrowing and diminished airflow [81]. AR in asthmatics is considered a consequence of repeated inflammatory episodes orchestrated through allergen sensitization, IgE-driven mast cell activation and massive inflammatory cell recruitment to airway compartment [44, 56]. Hypertrophy of the airway smooth muscle mass encircling conducting bronchi is an important pathological manifestation in this process. Interestingly, contemporary evidence implicates an extensive pathogenic role for ASM cells, which is attributed, in part to their ability to switch between multiple functional phenotypes [10, 82].

That mature ASM cells exhibit phenotype plasticity was first demonstrated by Halayko et al [83], where they showed that cultured airway myocytes transform into a hyperproliferative state upon exposure to mitogens. At the same time, these hyperproliferative cells exhibited a diminished contractile behavior in response to methacholine and decreased expression of contractile proteins including calponin, caldesmon, α-SMA, MLCK and smooth muscle-myosin heavy chain (sm-MHC) [83, 84]. Under *in vitro* conditions, asthmatic airway myocytes exhibit a hyperproliferative state and behave like an "inflammatory immune" cell compared to normal cells [69, 85]. Interestingly, ASM phenotype switching is a reversible process as removal of mitogenic mediators or serum-starvation re-establishes a hypercontractile and hypoproliferative phenotype, *in vitro* [10]. There exists literature evidence supporting the argument that phenotype modulation/maturation is not an artefact of cell-culture conditions, but indeed occurs under *in vivo* conditions. However, it remains yet to be definitive. This unique behavior of ASM cells can be partly attributed to their cellular heterogeneity, as shown by Sukkar *et al*, where they demonstrated an overlapping ASM cell population existing predominantly as synthetic/proliferative alongside a minor synthetic/non-proliferative phenotype [86]. Phenotypic plasticity provides ASM cells an ability to maintain a homeostatic ASM microenvironment and airway lumen diameter both in healthy and disease conditions. *In vivo*, ASM cells are believed to exist in an intermediary phenotype. When activated by exogenous stimuli, airway myocytes can contract, proliferate, migrate and release ECM proteins, cytokines, chemokines and growth factors [84, 85]. Multifunctional phenotypes of mature ASM cells are primarily driven by differentially

regulated expression of definitive phenotypic molecular markers [87]. For example, mature primary human bronchial ASM cells express contractile proteins such as calponin, SM22 and smooth muscle heavy chain (smHC). While proliferating ASM cells express proteins such as caldesmon and vimentin, they also express ECM proteins like fibronectin, collagen-1 and laminin [88]. Synthetic function of ASM cells is modulated by local inflammatory milieu; primarily TNF together with IL-1β induces ASM cells to express proinflammatory cytokines, chemokines, growth factors and adhesion molecules [87]. Also, ASM cells orchestrate leukocyte trafficking and asthmatic airway inflammation, thereby potentiating AHR and AR [87]. Different phenotype markers/proteins expressed by ASM cells are summarized in Table 1.1.5.

Table 1.1.5: An overview of markers expressed by ASM cells that determine ASM phenotype modulation and maturation

Contractile	**Proliferative**	**Synthetic**
↑ Calponin, SM-22, Desmin, α-SMA, sm-MHC, MLCK, Dystrophin-glycoprotein complex (DGC), Laminin-binding integrin (alpha7B)	↑Vimentin, h-Caldesmon, Fibronectin, Laminin, Collagen-1 Non-muscle myosin heavy chain, CD44, Alpha/beta-protein kinase C (PKC), Ki67 ↓	↑Adhesion molecules (ICAM-1, VCAM-1, CD44, LFA-1) ↑Chemokines (RANTES, Eotaxin, TARC, IL-8, Fractalkine, MCP-1, 2, and 3)

	Sm-MHC, MLCK, Calponin, α-SMA, caveolin-1, SERCA2	↑Cytokines (IL-2, IL-4, IL-5, IL-1β, IL-6, IL-13, IL-17, TNF, IL-9, IL-33, GM-CSF, IFNβ, TGFβ, TSLP and IP-10) ↑Growth factors (VEGF/vascular endothelial growth factor, PDGF, and stem cell factor/SCF) ↑ Matrix metalloproteinases (MMP-2, 3 and 9)

Source: *Adapted from [11, 83, 85, 89-91]*

Molecular mechanisms underlying regulation of ASM phenotype switching are diverse, some of which are discussed in the next section.

1.1.6 Mechanisms Regulating ASM Phenotype and Function

ASM dynamics and phenotypic reorganization of healthy & diseased airway myocytes is influenced by multiple factors including ECM, G-protein coupled receptor (GPCR) activation, inflammatory cytokines and chemokines, mitogens, reactive oxygen species, epigenetic changes, microRNAs [92], and presumably by neuromediators like

neurotrophins (NT) [93] and neurotransmitters like acetylcholine (ACh) [94], serotonin [94], dopamine [95] and glutamate [96].

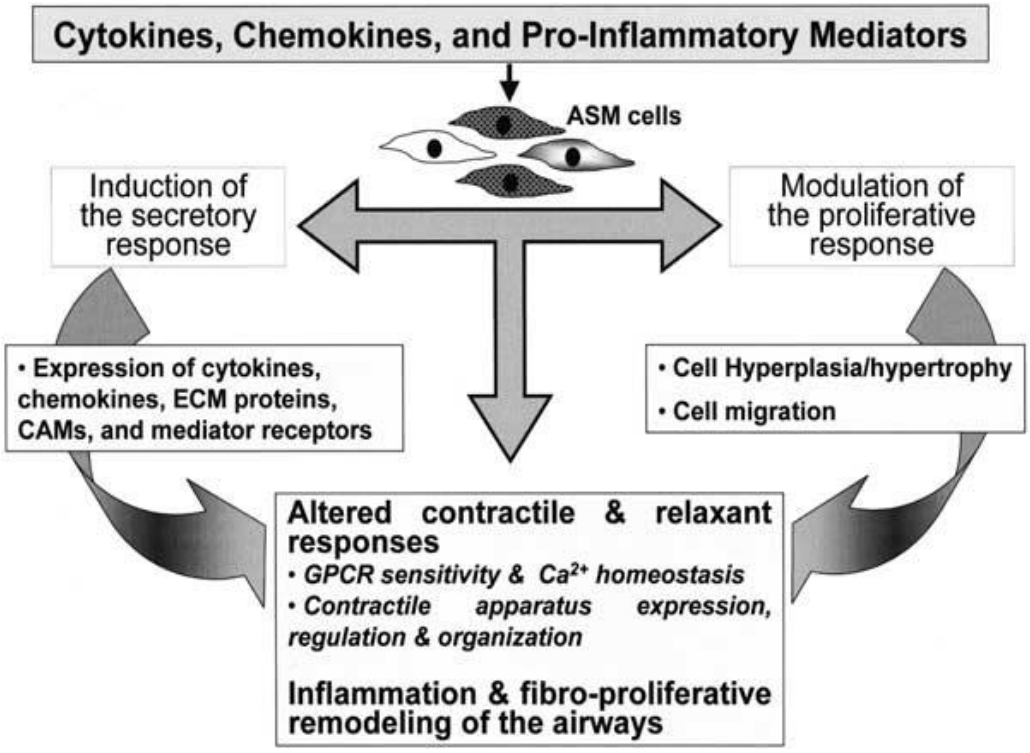

Figure 1.1.6: Model of the effects of pro-inflammatory mediators on functional plasticity of ASM cells, contributing to acute and chronic airway hyperresponsiveness and remodeling associated with bronchial asthma. In this model, inflammation drives functional responses in subsets of airway myocytes that differentially express inflammatory mediators and their receptors, growth factor receptors, CAMs, ECM proteins, and GPCRs. This process contributes both to structural changes in the airway wall (muscle layer thickening, increase in myofibroblasts, fibrosis, and recruitment of activated immuno-regulatory cells), and to airway hyperresponsiveness through intrinsic changes in contractile function, and responsiveness to bronchoconstrictors and bronchodilators. Abbreviation: ASM, airway smooth muscle. (Adapted from Halayko *et al* Respiratory Physiology & Neurobiology, Volume 137, Issues 2–3, 2003, 209 – 222)

Contractile: Altered airway narrowing mediated by excess contraction of airway myocytes contributes significantly to airway obstruction in pulmonary diseases like

asthma. As elucidated in figures 1.1.7 and 1.2.0, contractile phenotype of airway myocytes is coupled to Ca^{2+} release from the SR. Resting membrane potential of ASM cells ranges from -45mV to -70mV depending on the species, location of myocyte and the diameter of the airway [97-100]; and is regulated by membrane-bound potassium (K^+) channels [99, 101-103]. Following spasmogens binding to their receptors, transmembrane L-type voltage-operated Ca^{2+} channels (VOCCs) are activated, this initiating membrane depolarization of ASM cells. The process is further amplified via activation of inward chloride conductance channels and other non-selective cation channels (NSCCs) along with suppression of voltage-dependent K^+ channels, thereby activating airway myocyte contraction machinery and Ca^{2+}-dependent E-C coupling [104-106].

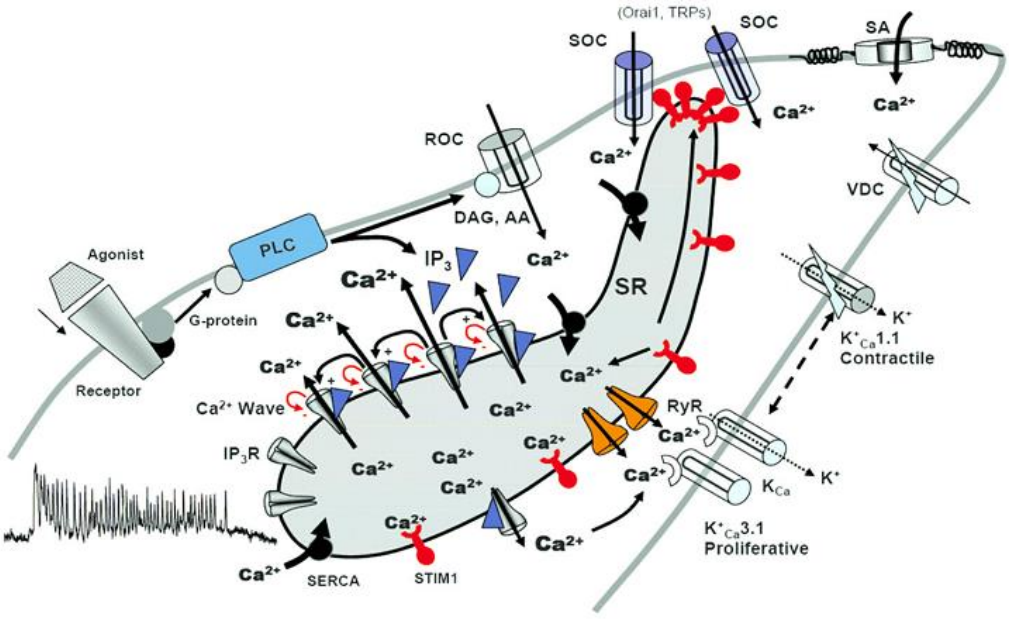

Figure 1.1.7 – Ion channels and their contribution to Ca^{2+} changes in airway smooth muscle cells - The binding of agonist to its receptor activates phospholipase C (PLC) via membrane G-proteins to synthesize inositol trisphosphate (IP_3) and diacylglycerol (DAG) from membrane lipids.

IP_3 (blue triangles) diffuses throughout the cell and binds to the IP_3R, sensitizing it to Ca^{2+}, which stimulates the opening of the receptor to allow the release of Ca^{2+} from the sarcoplasmic reticulum (SR). The released Ca^{2+} in turn stimulates (+ black arrow) Ca^{2+}-induced Ca^{2+} release from adjacent, sensitized IP_3Rs which results in the propagation of a Ca^{2+} wave. The released Ca^{2+} subsequently inactivates the IP_3R (− red arrow) and the Ca^{2+} is re- accumulated in the SR via Ca^{2+} pumps (SERCA). The repetitive release and uptake of Ca^{2+} results in Ca^{2+} oscillations. DAG and its derivatives (e.g. arachidonic acid, AA) move in the membrane to activate receptor-operated Ca^{2+} influx or channels (ROC) to replenish internal Ca^{2+} stores. During the Ca^{2+} oscillations, the Ca^{2+} store is partially depleted and this is sensed by STIM1 in the SR. STIM1 translocates through the SR membrane to aggregate with and activate store-operated Ca^{2+} entry or channels (SOC) (consisting of Orai1 or TRP proteins) to replenish the internal Ca^{2+} store. Ryanodine receptors (RyR) may release internal Ca^{2+} in the form of Ca^{2+} sparks to activate nearby K_{Ca} channels to induce membrane hyperpolarization. $K_{Ca}1.1$ channels may be replaced with $K_{Ca}3.1$ channels during cell proliferation to alter membrane voltage which may influence voltage-dependent Ca^{2+} channels (VDC). Stretch-activated channels (SA) are directly gated by physical stimuli (Reprinted from Perez-zoghbi *et al* Pulmonary Pharmacology and Therapeutics, 2009 October; 22 (5): 388-397).

Molecular mechanisms underlying ASM contraction and E-C coupling can be broadly categorized as MLCK-dependent and MLCK-independent, both of which are regulated by dynamic intracellular Ca^{2+}responses [23]. In MLCK-dependent regulation, bronchoconstrictors such as histamine, bradykinin and acetylcholine (ACh) released either by activated immune cells or neurons innervating the ASM bundles bind to their individual G-protein coupled receptors, particularly of G_q type [23]. On the contrary, ASM relaxation by bronchodilators such as isoproterenol (ISO) is mediated through activation of G_s-subtype of G-proteins, cyclic AMP (cAMP) and protein kinase A (PKA). Activation and translocation of G_q-proteins in the plasma membrane (PM) stimulates phospholipase-Cβ (PLC-β), which then produces secondary Ca^{2+} messengers molecules inositol-3-phosphate (IP_3) and 1,2 -diacylglycerol (DAG) via hydrolysis of phosphatidylinositol 4,5 – bisphosphate (PIP_2). While DAG binds to protein kinase C (PKC) in the cytoplasm and activates c-kinase-potentiated-phosphatase inhibitor 17 (CPI-17), IP_3 binds to IP_3R on the SR causing Ca^{2+} mobilization from the intracellular stores into the cytoplasm [23, 73]. Released calcium ions form complexes with calmodulin (CAM) protein, which then

activates MLCK causing MLC_{20} phosphorylation. This process triggers cycling of ATP-mediated actin-myosin crossbridges and ASM contraction. E-C coupling in ASM cells is negatively regulated by myosin light-chain phosphotase (MLCP). However, DAG/PKC/CPI-17 nexus functions as a potent MLCP inhibitor, thereby promoting contractility [73].

Other contractile mechanisms suggested to be involved include intracellular Ca^{2+} release due to activation of ryanodine receptors (RyRs) by cyclic ADP-Ribose (cADPR), RhoA-ROK-mediated inhibition of MLCP activity and non-canonical wingless integrase 1 (WNT)-Frizzled signaling [23, 107]. Ca^{2+} oscillations in ASM cells by RyRs are discussed further in the next section. Ras homolog gene family member A (RhoA) is a cytosolic small guanosine triphosphotase (GTPase) that exists in a GDP-bound form attached to an inhibitor complex. Gq-protein activation causes substitution of GDP to GTP on RhoA, a reaction catalyzed by guanine nucleotide exchange factors (GEFs). RhoA-GTP complex translocates to the PM and activates Rho-associated protein kinase (ROK/ROCK), which then inhibits MLCP function via phosphorylation of myosin phosphotase I (MYPT1). This stabilizes MLC_{20} in a phosphorylated state essential for ASM contraction [23, 73]. While RhoA and MLCK expression is increased in asthmatics, cytokines such as TNF, IL-1β and IL-13 augment GPCR-mediated contractile responses in ASM cells by inducing expression and activity of agonist-specific receptor and associated G-proteins [23, 73]. This partly answers the hypercontractile phenotype displayed by asthmatic ASM cells. Binding of non-canonical WNT ligands such as WNT5A to its specific receptors activates a β-catenin independent and IP3-mediated Ca^{2+} release from SR, which is implicated in smooth muscle

contraction and cell migration [107]. Recently, a MLCK and calcium-independent contractile mechanisms have been identified in ASM cells, which are mediated through p21-activated kinase 1 (PAK1), protein kinase C epsilon (PKCε) and caldesmon. However, the physiological relevance of these mechanisms in a disease state still remains elusive [23, 108].

***Proliferative**:* ASM cells in COPD and asthma are suggested to acquire an intrinsic abnormality in their proliferative behavior initiated by various mitogenic factors including growth factors, pro-inflammatory cytokines and contractile agents. Upon binding to their individual receptors, these factors activate a series of intracellular signaling cascades that converge downstream with activation of PI3K and MAPK molecules [23, 73]. While growth factor receptors have an intrinsic C-terminal tyrosine kinase (RTK) activity, spasmogen receptors are coupled to G-proteins [11, 23]. Members of MAPK superfamily that are activated during the process include MEK1 (MAPK/ERK Kinase 1), p38 and c-Jun-N-terminal-kinase (JNK). MEK1 phosphorylation leads to activation of extracellular signal kinase (ERK) -1, 2, which culminates with induction of JAK2 and STAT3, which are required for activation of cyclin-dependent kinases (CDKs), elongation factor 2 (E2F), hyperphosphorylation of retinoblastoma protein (Rb) and CyclinD1 synthesis [11, 23]. These chains of molecular events drive DNA synthesis and cell cycle progression from G1 to S phase, thus stimulating ASM mitogenesis [11, 23, 73]. PI3K activation leads to recruitment, phosphorylation and activation of various serine-threonine kinases such as protein-kinase B (PKB/Akt) and phosphoinositide-dependent kinase 1 (PDK1), which are intricately involved in cell survival and proliferation [23, 73]. Increase in ASM cell number

is negatively regulated by CCAAT/enhancer binding protein-α (c/EBP-α), a gene transcription factor that induces $p21^{waf1/cip1}$ synthesis and down-regulates cell cycle progression [11, 23]. Expression of c/EBP-α is significantly diminished in asthmatics, which favors increased proliferation [23, 73]. Recently, increased mitochondrial biogenesis together with reduced sarcoendoplasmic Ca^{2+} ATPase 2 (SERCA2) expression was proposed to be few other mechanisms associated with the process [109, 110]. However, their importance in disease still needs to be ascertained.

Synthetic: Contemporary evidence suggests that airway myocytes are capable of synthetic function, either as contractile or proliferating cells. Synthetic function of ASM cells orchestrates local inflammation, which might be an innate host defense strategy to eliminate allergens and other pathogens. The earliest evidence about secretory potential of ASM cells was provided by Berkman *et al*, when ASM bundles in bronchial biopsy sections from asthmatic patients stained positive for RANTES [61, 111].Cultured human airway myocytes, when stimulated with atopic serum or cytokines such as TGF-β, TNF and IL-1β, release an array of inflammatory mediators (as summarized in Table 1.1.5) and their receptors. Some of these secreted molecules participate in chemotactic recruitment and localization of MCs, eosinophils, neutrophils and other immune cells; others such as TGF-β, IL-1β PDGF and IGF also activate ASM cells in an autocrine/paracrine manner to induce ASM proliferation and express molecules such as connective growth factor (CTGF), GM-CSF, eotaxin-1 [61]. ASM cells express adhesion molecules ICAM-1, VCAM-1 and CD44 on their surface promotes T-cell adhesion, which then stimulates OX-40 ligand (OX40L) expression and IL-6 production. Also, reports suggest ASM cells

express various co-stimulatory molecules (MHC-II, CD40, CD80 and CD86) and toll-like receptors (TLRs) such as TLR3 and TLR4. However, it is still unclear on their role in perpetuating ASM inflammation [61].

Signaling mechanisms regulating immunomodulatory behavior in ASM cells remain poorly defined. Using selective chemical inhibitors, Maruoka *et al* and others showed that TNFα-induced RANTES production from ASM cells involves an interplay of MAPK, ERK and p38 signaling pathways [61, 112]. A similar sequence of signaling events are suggested in the expression of IL-6 and ICAM-1 [61]. Replacing TNFα with IL-1β as an inflammatory stimulus is supposed to activate ERK, p38 and JNK pathways, causing RANTES, eotaxin and GM-CSF release from ASM cells. STAT6 activation is believed to occur when IL-4 and IL-13 activate airway myocytes [61]. In addition to inflammatory stimuli, recruitment of transcription factors to gene promoter sites seems to be an additional level of gene regulation. For instance, Ammit *et al* have demonstrated that TNFα-mediated IL-6 expression is nuclear factor kappa B (NF-κB) – dependent, while RANTES promoter activation requires activator protein-1 (AP-1) and nuclear factor of activated T-cells (NF-AT) [61, 113].

Emerging evidence suggests epigenetic modifications in the chromosome and microRNAs (miRs) as important contributors to phenotype switching in ASM cells [114]. While histone deacetylases (HDACs) promote TNFα-induced IL-8, eotaxin-1 and IP-10 release from airway myocytes, methylation of CpG sites inside the GATA-binding domain

of CCR3 promoter region is speculated to correlate positively with increased CCR3 transcription in asthmatic ASM cells [114]. Similarly, HDAC inhibitors are proposed to exhibit an anti-proliferative effect and can negatively impact other aspects of AR including angiogenesis, airway constriction and synthesis of ROS. MiRs implied to be critically involved in ASM cell functions include let-7, miR-25, miR-26a, miR-133, miR-206 and miR-143/miR-145 cluster [114]. MiR-25 represses the function of kruppel-like factor 4 (KLF4), which is a transcriptional repressor of contractile protein smMHC. While miR-133 targets RhoA expression, miR-26a is suggested to promote ASM hypertrophy. Interestingly, miR profiles in tissues obtained from different animal models of asthma reveal differential expression of various miRs, indicating a completely different level of complexity in terms of gene regulation [114]. However, their impact in humans is still under investigation.

Cellular heterogeneity and intrinsic phenotypic differences in ASM cells accrued during airway inflammation contribute to AHR. Interestingly, dynamic regulation of intra and intercellular calcium responses across myocyte populations is widely regarded as key contributor to ASM phenotype switching between contractile, proliferative and synthetic states (explained in next section).

1.2.0 CALCIUM HANDLING IN ASM CELLS

A unique feature that distinguishes ASM cells from other smooth muscle tissues is their ability to generate Ca^{2+} oscillations dynamically, which can be briefly described as

rhythmic increase and decrease in intracellular Ca^{2+} concentrations following agonist stimulation. Earliest reports on the occurrence and mechanisms underlying Ca^{2+} oscillations in tracheal smooth muscle (TSM) cells were published by Liu and Farley[115], Prakash *et al* [116] and Kannan *et al* [117]. In their landmark investigations using isolated porcine TSM cells, they demonstrated that ACh-induced activation of muscarinic receptors caused repeated release of calcium from the internal stores, whose pattern was significantly distinct when compared to non-oscillatory inward Ca^{2+} currents initiated following caffeine stimulation. It is interesting to note that calcium oscillations in airway myocytes demonstrate significant heterogeneity depending on the species and location in the bronchial tree [118-120].

Based on the data obtained in several studies using various *in vitro* and *ex vivo* experimental approaches, baseline $[Ca^{2+}]_i$ in ASM cells is suggested to be between 100-200nM. In a non-oscillatory response, airway myocytes demonstrate a rapid and transient rise in cytosolic calcium levels upon agonist stimulation, where peak Ca^{2+} can increase up to 1µM. This is followed by an immediate decrease to an almost steady-state level, which plateaus over time depending on the agonist concentration and its binding affinity. In an oscillatory response, successive transient Ca^{2+} peaks occur, where amplitude of each peak is either sustained or decreases progressively till the agonist persists. In ASM cells, increase in $[Ca^{2+}]_i$ is due to membrane depolarization and simultaneous opening of various Ca^{2+} channels located on the plasma membrane and sarcolemma, thus favoring sudden influx of Ca^{2+} into the cytoplasm. On the other hand, decrease in $[Ca^{2+}]_i$ is dependent on the time taken for the clearance of calcium ions from the cytosol, which is in turn regulated by Ca^{2+}

uptake mechanisms is SR and mitochondria (which involve sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) channels and mitochondrial Ca^{2+} uniporter system), and channels that pump calcium out of the cell (briefly elaborated in the subsequent paragraphs). It should be noted that SR machinery takes a long time to refill Ca^{2+} stores, thereby causing significant delay in the return of $[Ca^{2+}]_i$ to its homeostatic equilibrium [118, 121]. Therefore, repetitive stimulations demonstrate decreased cellular responses. Interestingly, ASM cells do demonstrate significant heterogeneity in terms of cytosolic Ca^{2+} clearance, which partly defines the intracellular and intercellular heterogeneity in their responsiveness to a stimulus.

Emergence of *in situ* agarose-inflated *ex vivo* lung slice methodology had tremendously improved our current understanding on spatio-temporal changes in calcium oscillations observed in ASM cells. Ca^{2+} oscillations are spatially and temporally oriented and tend to originate at one end of cells and propagate in cyclic pattern as Ca^{2+} waves along the length of muscle bundle [122]. However, studies also suggest that waves can change direction, or can even originate at the center and propagate towards both ends of the cell [116]. This spatial heterogeneity is attributed to several factors including relative surface distribution of agonist receptors on the plasma membrane or number and relative concentration of Ca^{2+} influx channels in a localized region of the cell, both on the plasma membrane and sarcolemma[116]. Temporally, Ressmeyer et al showed that frequency of Ca^{2+} oscillations and their propagation velocity exhibited by HASM cells is completely dependent on the dynamics of the stimulation responsible for generating the signal [123]. Increased agonist concentration causes agonist binding strongly to its surface receptor, and

leading to repeated $[Ca^{2+}]_i$ release from stores, along with repeated Ca^{2+} reuptake in the SR [124, 125]. In fact, faster store-refilling rate is suggested to be a crucial mechanism underlying increased Ca^{2+} oscillation frequency and propagation velocity observed in certain smooth muscle types [122]. Interestingly, agonist concentration is suggested to have no influence on the peak Ca^{2+} levels demonstrated by cells following stimulation. Also, Ca^{2+} oscillation frequency, propagation velocity and amplitude are dependent on the type of agonist and presence of inflammation [118-120, 126, 127]. Ca^{2+} oscillation frequency in ASM cells is also dependent on the species. While Ca^{2+} oscillations in rats and humans occur at a slower frequency (~8 min^{-1}), mouse ASM cells demonstrate faster Ca^{2+} oscillations (~20-30 min^{-1} in response to contractile agonists such as ACh, 5HT, histamine or endothelin [119, 123, 128]. Similarly, studies done by Prakash *et al* and others have shown using porcine airway myocytes that ACh induces Ca^{2+} oscillations with a lower amplitude and take longer time to return to steady-state levels [116, 129]. Changes in the frequency of Ca^{2+} oscillations translates into increased contractile force by the muscle leading to increased ASM contraction, both in animals and humans [119, 123, 124, 126, 130]. One of the suggested mechanisms is the ability of Ca^{2+} oscillations to modulate the process of actin-myosin cross-bridge cycling to a constant "latch" state, which enables ASM strips to maintain their contractile force and tone [131]. Interestingly, there seems to be ~200-500ms time delay in terms of agonist-induced increase in $[Ca^{2+}]_i$, and its translational into a contractile force observed in ASM tissue, as measured using isometric recordings of tracheal strips or decreased bronchial lumen diameter in a lung slice preparation [118, 122]. Over the years, a number of mathematical models were developed to explain this phenomenon, which suggest this difference can be attributed to various

factors intrinsic to airway myocytes. They include dynamics of increased Ca^{2+} (transient vs. sustained), maximal peak Ca^{2+} achieved during a stimulation, and kinetics involved in the intracellular recruitment and sequestration of calmodulin, which in turn activates MLCK-mediated contractile apparatus [118]. However, all these likelihoods require further investigations to validate their importance and relevance to ASM physiology.

Ca^{2+} oscillation frequency and signaling in ASM cells is dependent on 3 important parameters: (1) Ca^{2+} sequestration into and release from internal stores, (2) influx and efflux of Ca^{2+} ions in and out of the cells and (3) sensitivity of intracellular proteins to changes in Ca^{2+} levels [115, 116, 125]. While events (1) and (2) are central for maintaining Ca^{2+} homeostasis inside the cell, (3) determines downstream signaling and eventual effect on ASM function. Intracellularly, Ca^{2+} is mainly stored inside the SR sequestered with proteins calreticulin and calsequestrin. As described in previous sections and figure 1.2.0, in airway myocytes, agonist-binding to their transmembrane G-protein coupled receptors causes membrane depolarization, E-C coupling and influx of Ca^{2+} ions through VOCCs. This leads to $G\alpha_q$-mediated PLCγ activation and generation of secondary calcium messengers, IP_3 and DAG. IP_3 binds to IP_3-receptors on sarcolemma causing channel-opening and Ca^{2+} efflux locally from the SR into the cytoplasm. Released $[Ca^{2+}]_i$ in turn activates adjacently located IP_3Rs leading to further release of Ca^{2+} ions, thereby initiating Ca^{2+} waves across the cell. At the same time, released Ca^{2+} also triggers neighboring ryanodine receptors (RyR), which further augments Ca^{2+} oscillations in ASM cells [124]. Depletion of Ca^{2+} stores initiates a feedback inhibition loop, blocks IP_3Rs and RyRs, eventually terminating Ca^{2+} release from the stores and activation of SERCA channels

involved in calcium reuptake from the cytosol. In another mechanism, Ca^{2+} oscillations are suggested to occur directly through RyR-dependent repeated Ca^{2+} release and uptake mechanism from the stores in an IP_3-independent manner [117]; albeit similar to mechanisms operative in striated muscle fibers such as skeletal and cardiac muscle. In skeletal muscle, L-type calcium channel ($Ca_v1.1$) physically interact with juxtaposed RyR1 isoform following membrane depolarization, leading to Ca^{2+} release from stores. On the contrary, influx of Ca^{2+} in cardiac muscle occurs through $Ca_v1.2$ channels, which in turn activates RyR2 isoform on the SR and culminates as release of stored Ca^{2+}, a phenomenon known as calcium-induced calcium release (CICR) [132]. Du *et al* demonstrated that rat ASM cells express functional $Ca_v1.1$. $Ca_v1.2$, $Ca_v1.3$, RyR1, RyR2 and RyR3 proteins, while expression of RyR isoforms was also determined in porcine and human ASM cells [117, 133, 134]. Incidentally, the functional role of RyR isoforms and role of RyRs in regulating agonist-induced Ca^{2+} oscillations and subsequent airway responses is debatable. Recent reports by Tazzeo *et al* and Ressmeyer *et al* suggest RyRs do not directly mediate airway contractile and dilatory responses in human and bovine ASM upon exposure to contractile agonists MCh and histamine, but are only involved in the expulsion of $[Ca^{2+}]_i$ from the cell [123, 135]. These observations were supported by Bai *et al* using murine lung slices, where authors showed that inhibition of RyRs using ryanodine did not have any noticeable effect on MCh-induced Ca^{2+} oscillations and airway contractions. On the contrary, these responses were largely IP_3-dependent, as antagonizing IP_3R induced bronchodilation in a dose-dependent manner [123, 136]. On a related note, Croisier *et al* suggest RyR sensitization substantially increases mean cytosolic Ca^{2+} levels and induces significant store-operated calcium entry [137]. While these studies do point out RyRs role

under normal physiological conditions, they do not shed any light on their importance in chronic inflammatory conditions, thus warranting further investigations. It should be noted that HASM cells demonstrate increased RyR, IP3 and SERCA2 protein expression, when exposed to pro-inflammatory cytokines IL-4 and IL-13 [138]. Additional factors that regulate Ca^{2+} oscillation frequency include concentration of intracellular signaling molecules, which include cAMP, cGMP, PI3Kγ and cyclic ADP-Ribose (cADPR) [139-142]. Cyclic ADPR is a secondary calcium messenger derived from nicotinamide adenine dinucleotide (NAD) due to ADP-ribosyl cyclase activity of transmembrane CD38 receptor. Additionally, cADPR is suggested to function as an endogenous RyR ligand modulation Ca^{2+} mobilization from these channels [143]. Recent studies by Deshpande *et al* and others suggest CD38-cADPR signaling in airway myocytes is an important contributor of AHR and altered lung function, as was observed in HASM cells and *in vivo* mouse models of airway inflammation [144-147]. It is interesting to note that reducing/inhibiting the agonist-induced Ca^{2+} oscillation frequency in airway myocytes using bronchodilators such as formoterol, nitric oxide or chloroquine induces significant airway relaxation or bronchodilation [123, 140, 148]. Taken together, these observations demonstrate the need for better understanding about Ca^{2+} oscillations in ASM cells as these cyclic responses might define the heterogeneity in smooth muscle responses, as observed within the tissue and within different species. Also, Ca^{2+} oscillations have to be diligently evaluated and assessed to explore their potential as possible therapeutic intervention in the treatment of obstructive airway disorders.

Cytoplasmic Ca^{2+} binds to and activates a series of intracellular protein cascades and affects multiples ASM functions, some of which are detailed in previous sections. Considering limited capacity of internal stores, inward Ca^{2+} flow from the extracellular compartment is essential to ascertain uninterrupted Ca^{2+} signaling and prolonged excitation/contraction coupling in ASM cells. Calcium replenishment into the SR and other cellular organelles is regulated by SERCA and bidirectional sodium-calcium exchanger (NCX) channels (Figure 1.2.0) [110, 124]. Additionally, emptying of internal stores stimulates opening of store-operated calcium channels (SOCCs) on the PM, a process termed as capacitative or store-operated Ca^{2+} entry (SOCE) [107, 110]. Low Ca^{2+} levels inside the SR cause punctated oligomerization of stromal-interacting molecule 1 (STIM1) on the SR membrane. STIM1 interacts with Orai1 and Orai3 proteins on the PM leading to Ca^{2+} inflow [107, 110]. Another family of proteins that function as classical SOCCs are transient receptor potential C (TRPC) channels (Figure 1.2.0). Recently, studies have identified that expression of SOCE members (STIM1/Orai1/TRPC) is drastically elevated under inflammatory scenarios, both in cultured HASM cells and *in vivo* animal models for allergic asthma [107, 110].

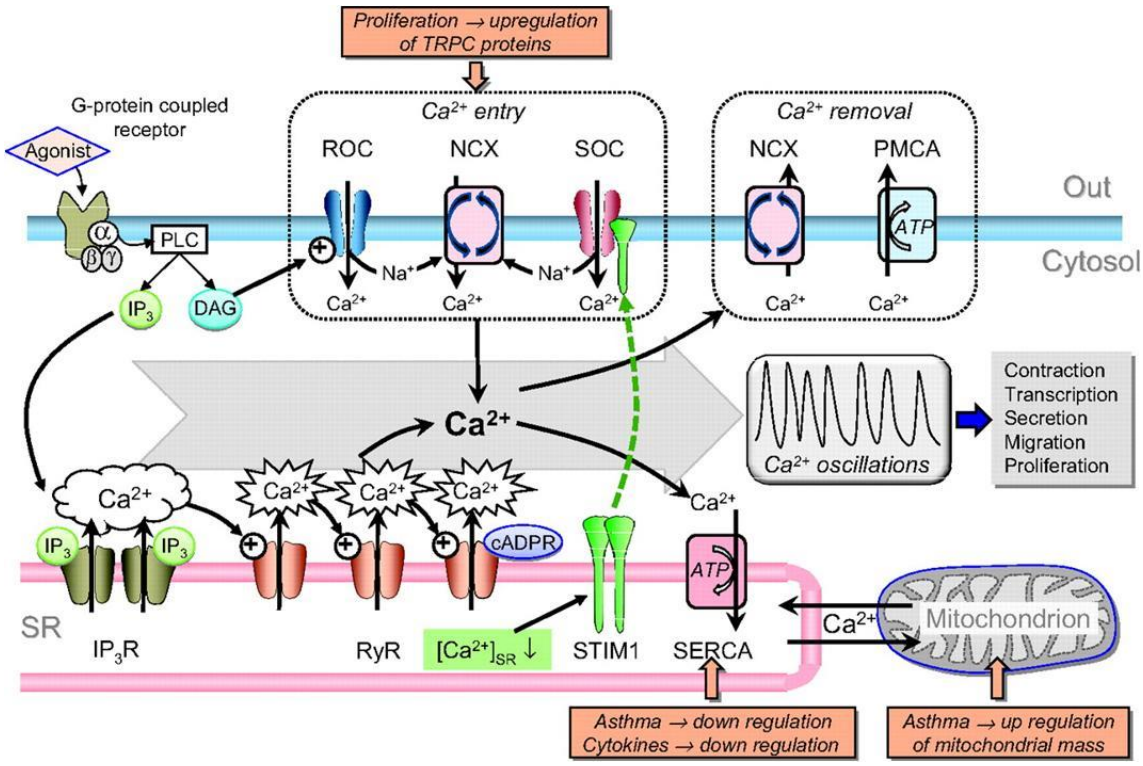

Figure 1.2.0: Ca2+ mobilisation pathways in airway smooth muscle (ASM). Binding of an agonist to a G-protein-coupled receptor activates phospholipase C (PLC) to generate diacylgycerol (DAG) and inositol trisphosphate (IP3). DAG activates receptor-operated channels (ROCs), eliciting Na+ and Ca2+ entry, while IP3 activates clusters of IP3 receptors (IP3Rs) on the sarcoplasmic reticulum (SR), causing Ca2+ release which may activate adjacent ryanodine receptors (RyRs) to increase the frequency of sparks. RyRs may also be activated or potentiated by cADP ribose (cADPR). Store depletion is sensed by stromal-interacting molecule 1 (STIM1) within the SR, which translocates to and activates store-operated channels (SOCs). The elevation in subplasmalemmal [Na+] resulting from activation of non-selective ROCs or SOCs may be sufficient to drive reverse-mode operation of Na+/Ca2+ exchanger (NCX), leading to Ca2+ entry. Much of the Ca2+ entering the cell and released from stores may be sequestered by the superficial SR through sarcoendoplasmic Ca2+ ATPases (SERCAs) and mitochondria. Cycling between SR Ca2+ uptake and release mechanisms leads to Ca2+ oscillations. Ca2+ is removed from the cell by the plasma membrane Ca2+ ATPase (PMCA) and normal operation of the NCX. TRPC, transient receptor potential channel (Reprinted from Mahn *et al* Thorax 2010 Jun; 65(6):547-52)

$[Ca^{2+}]_i$ flux is also mediated by an alternative pathway called receptor operated calcium entry (ROCE), which acts independent of Ca^{2+} release from internal reserves [107, 110]. Receptor-operated calcium channels (ROCCs) are activated directly by specific

ligands or indirectly via binding of secondary calcium messengers such as DAG. Some notable ROCCs include TRP channels (TRPC and TRPA), arachidonic-acid regulated (ARC) channels and ATP-gated P2X receptors [107]. Available *in vitro* and *in vivo* experimental evidence suggest some of these Ca^{2+}-permeable channels can potentiate methacholine-induced bronchial responsiveness and ASM-related functions. However, lack of appropriate pharmacological tools is a major impediment to discern their involvement in modulating ASM responses [107]. ***In my study, I have identified N-methyl-D-aspartate receptors (receptor for neurotransmitter glutamate) as another important ROCC in ASM cells, whose functional importance forms the crux of this thesis.***

1.3.0 NEUROTRANSMITTERS IN AIRWAYS

Pathogenesis of allergic diseases, asthma included, involves a complex interplay of innate and acquired inflammatory immune responses, which are in part regulated by nerve endings innervating lungs. Autonomous nervous system comprised of adrenergic (sympathetic), cholinergic (parasympathetic) and NANC nerves release specific neuromediators, which in turn control pulmonary functions including mucus gland secretions, bronchial responsiveness and airway inflammation. For instance, ACh released by the parasympathetic nerves, acts through muscarinic receptors expressed on both structural and immune cells [149, 150]. Muscarinic receptors are metabotropic GPCRs broadly classified into excitatory (M1, M3 and M5) and inhibitory (M2 and M4) subtypes depending on their coupling to G-proteins [149, 150]. While ASM cells express M2 and

M3 subtypes, expression of M1, M3, M4 and M5 receptors was detected on eosinophils, macrophages, neutrophils and MCs. M3 receptor activation is required for ASM contraction, it also increases mucus secretion from glands, leukotriene B_4 (LTB_4) release from macrophages and eosinophil activation [149]. Alternatively, ACh binds to nicotinic receptors and co-ordinates an anti-inflammatory response in the airways [149].

The pulmonary adrenergic system is relatively underdeveloped in humans and is dependent primarily on circulating catecholamines such as epinephrine and norepinephrine. These catecholamines activate a specific family of adrenergic receptors expressed on ASM cells and other structural cells, thereby causing airway relaxation. Also, adrenergic receptor activation prevents eosinophil degranulation, inhibits histamine release from MCs, blocks acetylcholine secretion from parasympathetic system and stimulates synthesis of proinflammatory molecules TNF, IL-1β and IL-6 from macrophages [149]. Examples of NANC mediators, which are either inhibitory or excitatory, include NO, VIP, substance P (SP), neurokinin A (NKA), and calcitonin gene-related peptide (CGRP) [149].

A role of neurogenic mechanisms in maintaining ASM tone, caliber and associated physiology has received relatively lesser attention. Traditionally, neural communication in the peripheral organs has been considered as being one-way traffic with innervated nerve endings releasing different neuromediators, which instigate paracrine signaling by binding to their specific receptors expressed on surrounding immune and structural cells (Fig 1.3.0). Emerging evidence suggests existence of an opposite phenomenon; epithelial cells, ASM

cells and immune cells such as lymphocytes, DCs, neutrophils, and mast cells release various neuromediators, which include ACh [149, 151, 152], serotonin [94, 153], γ-amino butyric acid (GABA) [154], brain-derived neurotrophic factor (BDNF) [93], and NGF [93]. Furthermore, expression of receptors for these neuronal molecules in various non-neuronal compartments, including the immune system, suggests a bidirectional interaction that might contribute to pathophysiology of various lung diseases, including asthma. Glutamate is one such neuromediator whose role as an excitatory neurotransmitter had undergone a substantial revision in recent years.

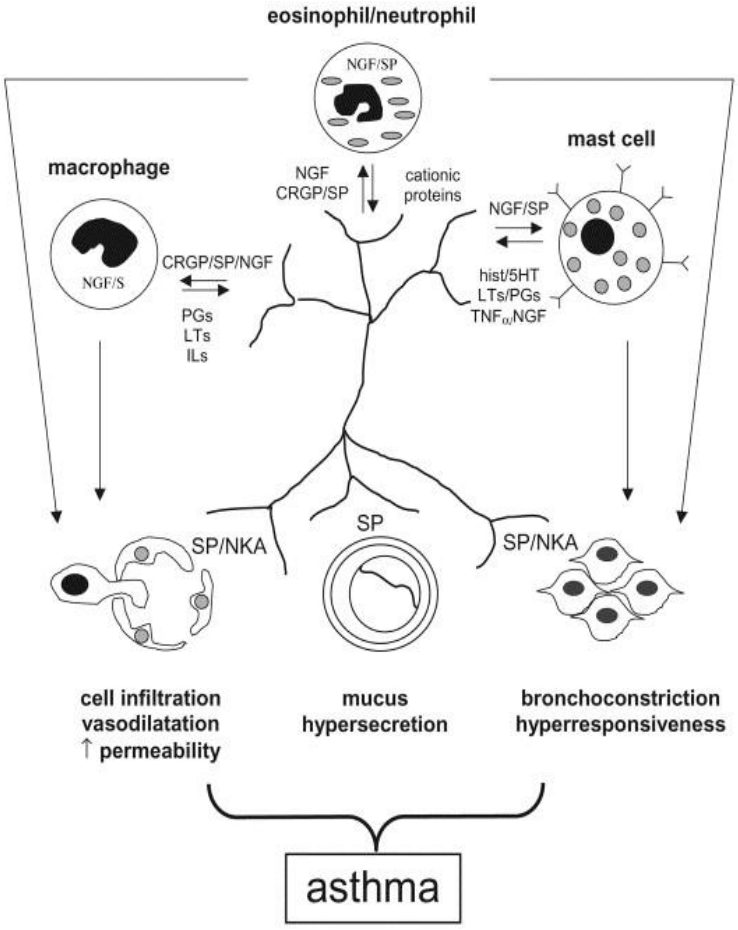

Figure 1.3.0: The possible interactions between inflammatory cells and excitatory NANC nerves in asthma leading to bronchoconstriction, airway hyperresponsiveness, mucus secretion, oedema

formation and cellular infiltration. (Abbreviations: SP: substance P; NKA) – Reprinted from Kraneveld *et al* International Immunopharmacology, Volume 1, Issues 9–10, 2001, 1629 – 1650.

Synthesized *de novo* by astrocytes in the brain, glutamate is the most abundant non-essential dicarboxylic amino acid involved in neuronal synaptic transmission and is essential for brain development and its normal physiological functioning [155, 156]. In addition, glutamate is essential for protein catabolism and anabolism, glutathione synthesis, ammonia removal, and formation of key citric acid cycle intermediates like α-keto glutarate, and inhibitory neurotransmitter γ-aminobutyric acid (GABA) [157]. Presence of extremely low extracellular concentrations of glutamate is a critical requirement for normal glutamate functioning. Under physiological conditions, interstitial glutamate concentrations in the brain and cerebrospinal fluid (CSF) are tightly regulated; while 2-5mM glutamate concentration exists intracellularly in neurons and astrocytes, extracellular levels are in the range of 1-2μM. In CSF, glutamate concentration is ~1μM [155]. In contrast, glutamate in peripheral organs is primarily obtained from dietary sources, whose levels in plasma/blood are between 30-80μM [155, 158, 159]. However, glutamate outside the CNS is endogenously synthesized and released by various immune cells including neutrophils, DCs and macrophages [160-163]. Evidence from our lab confirms that even eosinophils constitutively release glutamate (data not shown). Even mature DCs, upon formation of an immunosynapse with T-lymphocytes, release copious amounts of glutamate [160]. While glutamate promotes/directs migration of human neutrophils to sites of inflammation following injury or infection [164], neutrophil-derived glutamate regulates permeability of endothelial cell barrier lining the vasculature [165]. Glutamatergic signaling promotes histamine exocytosis from MCs and regulates DC

function [166]; while glutamate induces ECM adhesion, migration and cytokine secretion from T lymphocytes [160, 167]. Interestingly, DCs and T lymphocytes express both metabotrophic and ionotrophic glutamate receptors[159, 167]; while eosinophils are presumed to be involved in the maintenance of a Th2 polarized state inside the lungs during allergic diseases by inducing apoptosis of Th1 cells, via induction of indoleamine 2, 3-dioxygenase (IDO), a rate-limiting step involved in degradation of tryptophan into kynurenines [44, 168]. These observations suggest glutamate-mediated neuroregulatory axis is an important contributor of inflammatory responses within the local airway microenvironment that needs further verification [159, 169-172].

In various neurodegenerative pathologies such as stroke, Alzheimer's disease and meningitis, deregulated glutamate homeostasis outside the cell cause severe neuronal damage through a phenomenon known as "excitotoxicity" [155]. Surprisingly, increased glutamate extravasation into extracellular spaces has been documented as an important pathological event in patients suffering with diseases like rheumatoid arthritis, HIV and cancer [158, 173]. Glutamate toxicity associated with increased plasma glutamate levels is suggested in lung disorders such as acute lung injury (ALI) [173]. ***Immune cells are principal regulators of airway responses whose function is induced by glutamate. Also, functional glutamate receptors exist in the lungs and airways. This suggests a possible involvement of glutamatergic signaling in the regulation of lung and airway physiology, which forms the fundamental rationale for this thesis.*** Principal receptors participating in glutamate signaling are outlined in the next section.

1.4.0 GLUTAMATE RECEPTORS

Glutamatergic signaling is mediated by two classes of glutamate receptors: metabotropic (mGluRs) and ionotropic (iGluRs). While mGluRs are seven transmembrane GPCRs, iGluRs are ligand-gated ion channels further divided into NMDA receptors (NMDA-R) and non-NMDA receptors (Kainate and AMPA receptors) [155].

1.4.1 Metabotropic Glutamate Receptors (mGluRs)

Belonging to family C of Ca^{2+} sensing 7-transmembrane GPCRs, mGluRs are evolutionary conserved across species and distributed ubiquitously, both in CNS and peripheral organs [158, 174]. Located in the vicinity of synaptic cleft, mGluRs are involved in neurotransmission, neuronal development, cellular proliferation, and generation of long-term potentiation (LTP), modulation of neurotransmitter release from presynaptic neurons, memory, anxiety and depression. In addition, mGluRs are presumed to be essential for trafficking of iGluRs and modifying their responses [175]. Till date, 8 different mGluRs are discovered (mGluR1-8), which are further classified into 3 major sub-groups based on their structural configuration, sequence homology, agonist-binding affinity and intracellular signaling mechanisms; group I (mGlu1 and 5), group II (mGlu2 and 3) and group III (mGlu4, 6, 7 and 8) [158, 175]. Activation of group I receptors engages G_q subunits on the C-terminus of the receptor and causes synthesis of IP_3 and DAG [174]. Further, this results in $[Ca^{2+}]_i$ mobilization from internal reserves and PKC activation, thereby activating downstream JNK, MAPK/ERK, and mTOR signaling leading to transcriptional regulation of genes involved in cell cycle and cellular growth/survival

[174]. On the contrary, group II and III are coupled to inhibitory $G_{i/o}$ subunits whose activation leads to decreased intracellular cyclic-adenosine monophosphate (cAMP) levels [174].

Current literature suggests mGluRs play a pivotal role in the maintenance of organ homeostasis outside the CNS. On the tongue, ability to distinguish "*umami*" taste in food is the most recognized peripheral function of mGluRs [158]. Additionally, mGluRs are involved in the regulation of gastro-esophageal reflux, secretion of gastric juices, maintenance of mucosal integrity inside the duodenum and colon, motility of intestinal villi and microbiota composition [158]. Other non-neuronal contributions of mGluRs include balancing renal electrolyte transport, and regulating the production of various endocrine hormones such as pituitary, adrenocorticotrophin, oxytocin, prolactin, and catecholamines [158]. The presence of metabotropic glutamatergic system is considered critical for optimal glucagon/insulin response to glucose inside pancreas, T-lymphocyte maturation and activation in thymus, prevention of oxidative damage in endothelial cells, protection of vascular endothelial barrier integrity and maintenance of bone mass density [158]. Also, mGluR5 and mGluR3 are expressed in lungs; their physiological role in still unidentified [158].

1.4.2 Ionotropic Glutamate Receptors (iGluRs)

Critical for mediating rapid excitatory neuronal impulses, iGluRs are encoded as 18 different protein subunits, which assemble as membrane-integrated tetrameric

complexes permeable to cations such as Ca^{2+}, upon glutamate binding. Widely implicated in various neurological pathologies, iGluRs mediate a broad spectrum of physiological events in the CNS, spinal cord, retina, bone, and several other peripheral organs including lungs and airways. Structurally, all iGluRs are similar comprised of 4 distinct regions; an extracellular N-terminal domain (ATD) essential for receptor assembly and trafficking to membrane, agonist-binding domain (LBD), 4 pore-forming transmembrane regions (TMD), and an intracellular C-terminal domain (CTD). Agonist binding to its domain causes a conformational shift in receptor subunit structure and change in TMD orientation leading to formation of a pore-like channel, which facilitates cation influx into the cells. Based on the subunit configuration and specificity of pharmacological agonists activating these receptors, iGluRs are classified into kainate, a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA receptors, which are detailed below.

1.4.2.1 Kainate Receptors

As their name suggests, "kainate receptors" are selectively activated by kainic acid (kainate), a naturally-occurring glutamate analog derived from marine seaweed. Other pharmacological agents that can activate and partially desensitize these receptors include domoate and 5-tert-butyl-4-isoxazolepropionic acid (ATPA) [176, 177]. Distributed ubiquitously inside cerebellum, hippocampus and other regions of brain, human kainate receptors are comprised of 5 different subunits namely GluR5, GluR6, GluR7, KA1 and KA2 (GluK1-5) [176, 177]. Interestingly, GluR5-7 subunits combine to form either homomeric or heteromeric functional receptors. On the contrary, KA1 and KA2 subunits

exist only in heteromeric combinations with GluR5-7 [176-178]. Although kainate receptors remain the most elusive iGluRs, available research suggests these receptors coexist with NMDA and AMPA receptors and participate in post synaptic neurotransmission as well as presynaptic release of neurotransmitters glutamate and GABA [176-178]. Dual signaling ability upon glutamate binding is an important characteristic feature of kainate receptors, as they can function as conventional ion channels or activate a non-canonical intracellular signaling pathway via G-protein activation [177]. Other suggested functions of kainate receptors that still require further investigation include axonal growth and guidance during development, synaptogenesis and regulation of dorsal root ganglion responses [177, 178]. While the involvement of these receptors in brain diseases such as epilepsy, schizophrenia, mental retardation and mood disorders remains inconclusive, their presence and functional role in peripheral tissues still remains questionable.

1.4.2.2 AMPA Receptors

Activated specifically by AMPA, a synthetic pharmacological glutamate analog, AMPA receptors (AMPA-Rs) are the most extensively investigated non-NMDA iGluRs that predominantly mediate rapid/fast glutamatergic postsynaptic excitatory responses in the brain and other parts of the nervous system. Also termed as quisqualate receptors, human AMPA-R were discovered in 1982 and are comprised of 4 different subunits (GluA1 - A4). These multisubunit protein complexes exist on the plasma membrane as either quaternary homomer or as heteromeric assemblies channelizing cation flow in the

presence of glutamate [179]. Similar to NMDA-R, subunit composition determines the biophysical and cellular outcomes of a particular AMPA-R [179]. Glutamate released from excitatory neurons binds to AMPA-Rs located on the postsynaptic membranes, which opens ion channel pore leading to influx of cations, primarily Na^+ and K^+ ions [180]. Influx of Na^+ ions causes a momentary membrane depolarization and generation of an excitatory postsynaptic potential (EPSP) [180]. The process is further compounded by the activation of voltage-gated Na^+ and K^+ channels that exist in the vicinity of AMPA-R. All the EPSPs, thus generated during the process, combine to generate an action potential, which culminates the synaptic signal transmission [180].

A few AMPA-Rs are also permeable to Ca^{2+}flux; only receptors lacking GluA2 subunit can accomplish it [180, 181]. While AMPA-R numbers on the postsynaptic membranes are suggested to define synaptic strength and transmission efficiency, these receptors are essential for maintenance of synaptic structure, spatial memory performance and proper functioning of neuronal circuits and cognitive abilities [181]. Use of selective pharmacological antagonists such as perampanel made it possible to appreciate the role of AMPA-R as promising therapeutic targets against epilepsy and related seizures [180, 181].

Pathologically, changes in receptor numbers and decreased GluA2 subunit expression causing alteration of AMPA-R composition and elevated Ca^{2+} inflow are proposed to be some possible mechanisms underlying diseases such as amyotrophic lateral sclerosis (ALS), Rett syndrome, dementia associated with impaired cognition, ischemia

and Alzheimer's disease [182]. Unfortunately, clinical trials with AMPA-R antagonists or agents targeting adenosine deaminase acting on RNA2 (ADAR2; an enzyme required for proper editing of GluA2 to prevent its degradation) have yielded unpromising results, which suggests direct modulation of AMPA-R activity is detrimental and requires an alternative approach [181]. Peripherally, AMPA-R subunits, both at mRNA and protein level, were isolated from various tissues such as heart, pancreas, ovary, testis, and kidney [173]. While preliminary investigations in animals reveal that these receptors are functional in some of these peripheral sites, we are quite far from understanding their relevance in human physiology.

1.4.2.3 NMDA Receptors

NMDA-R are heteromeric, ligand-gated, multiprotein complexes that form intrinsic membrane-bound ion channels and are non-selectively permeable to cations, particularly Ca^{2+}, but also Na^{+} and K^{+}. In the nervous system, NMDA-R exist at the excitatory synapses, are activated by glutamate binding and mediate neuron signaling [183]. NMDA-R are unique in their ability to bind multiple ligands for activation and exhibit voltage-dependent activation and slow deactivation kinetics, a characteristic feature crucial for the regulation of long term potentiation of memory and synaptic plasticity in brain [183]. NMDA-R structure, subunit organization, and its physiological and pathological attributes are elaborated in the later sections.

1.5.0 STRUCTURE AND PHYSIOLOGICAL ROLE OF NMDA RECEPTORS

Named owing to their selective affinity to NMDA, a synthetic pharmacological glutamate analog, NMDA-R are remarkably diverse non-selective cation channels first discovered in the early 1980s as proteins containing binding sites for phencyclidine and ketamine used as possible therapeutic drugs for schizophrenia. Since then, there has been tremendous augmentation in our understanding about these receptors in terms of their biophysical properties and numerous physiological (neuronal growth, memory, migration and synaptic plasticity) and pathological (epilepsy, seizures, HIV-dementia, Parkinson, Alzheimer's and Huntington) implications.

1.5.1 NMDA Receptor Complex: Structure and Subunit Composition

Structural topology of NMDA-Rs is very similar to other iGluRs (figure 1.5.2.2). Crystallographic studies reveal each NMDA-R subunit is composed of an extracellular ATD followed by an agonist binding domain (ABD) comprised of 2 distinct and discontinuous regions (S1 and S2). While S1 domain starts after the ATD and ends before the TMD, S2 domain is present after the TMD and links M3 and M4 domains as an extracellular loop. TMD is comprised of 3 transmembrane regions (M1, M3 and M4) and a re-entrant loop (M2 region) that forms the pore. The fourth region is the intracellular CTD [184, 185].

ATD, consisting of ~380-400 aminoacids, is made of 4 tandem globular domains and is essential for receptor assembly on the membrane surface. ABD (~300 aminoacids length) required for binding agonists' glutamate and glycine is structured as a bi-lobed clamshell or Venus flytrap like module with the ligand positioned in the central cleft between both lobes. Re-entrant loop determines Ca^{2+} permeability of the channel. The terminal CTD region is made of multiple short length motifs and interacts with various intracellular proteins involved in receptor trafficking, membrane anchoring and further signaling [184, 185].

Presence of multiple subunits allows the possibility of various NMDA-R subunit compositions, thereby giving rise of multiple receptor subtypes' each with a distinct biophysical property and functional significance. To date, seven NMDA-R subunits, divided into 3 structural groups, have been identified (figure 1.5.2.1): obligatory NR1 subunit (comprising 8 splice variants encoded by GRIN1 gene), 4 modulatory NR2 subunits (A, B, C and D, encoded by GRIN2A-D genes) and 2 inhibitory NR3 subunits (A and B, encoded by GRIN3A and B genes respectively) [186].

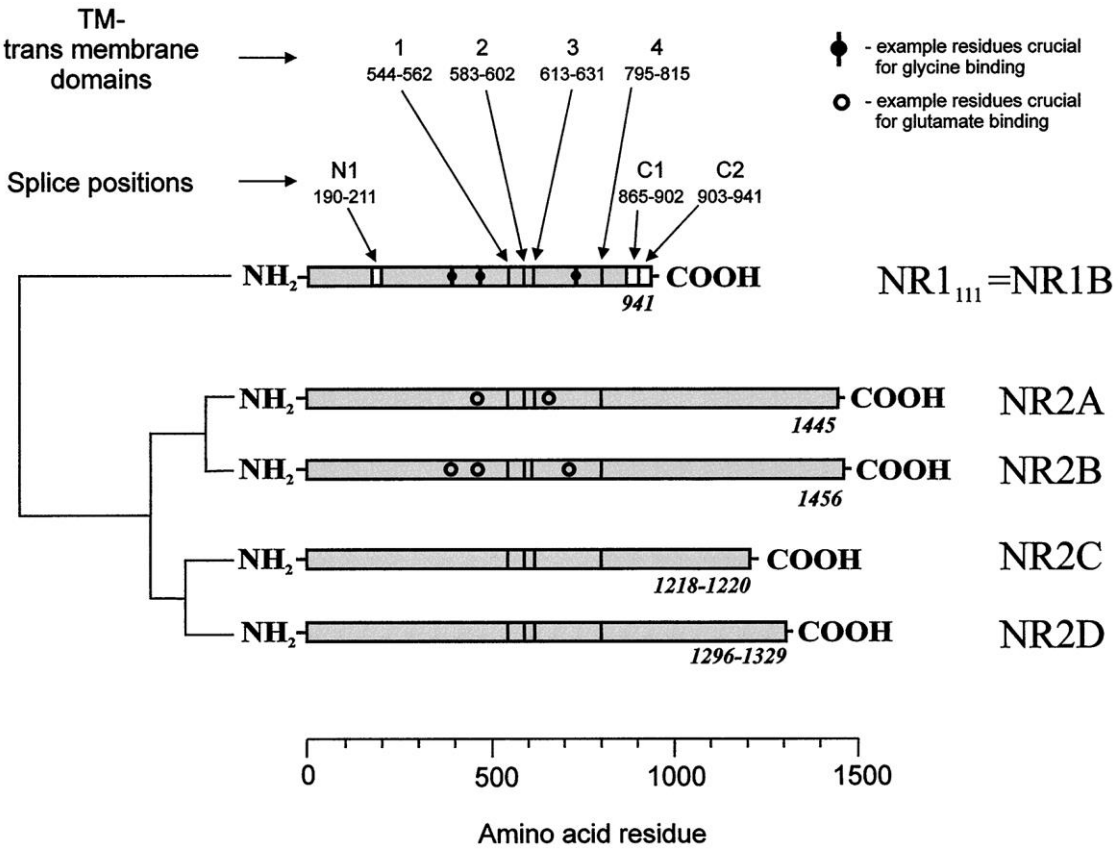

Figure 1.5.2.1 – Schematic presentation of the NMDA receptor subunit family. The tree indicates the degree of homology. For clarity, a NR1 receptor subunit splice variant having all alternatively spliced exons, i.e., N1 (exon 5, 21 amino acids), C1 (exon 21, 37 amino acids), and C2 (exon 22, 38 amino acids), is shown (Zukin and Bennett, 1995). Therefore, the TM and point mutation positions are numbered taking into account the presence of the N1 exon, i.e., positions are shifted by 21 amino acids, compared with original publications. Amino acid residues crucial for the binding of glutamate at NR2A or NR2B subunits and that of glycine at NR1 subunits are shown. The marks (closed and open circles) indicate residues at which point mutations resulted in at least 100-fold decreases in the affinity of glycine or glutamate. Glutamate binding sites also exist in the NR2C and NR2D subunits, but their exact locations have not been defined and are therefore not indicated in the scheme (Reprinted from Danysz *et al* Pharmacological Reviews 50 (4) 597-664, 1998[187]).

Evolutionarily conserved across species, all the NMDA-R subunits demonstrate a similar domain organization. *In vivo,* a functional NMDA-R assembles as a heterotetramer comprised of 2 obligatory NR1 subunits and a dimer of either NR2 and/or NR2/NR3

subunits (figure 1.5.2.2). While NR1/NR2 (A-D) tetramer combination is a general occurrence, NR1/NR2/NR3 arrangement is less common and these channels exhibit reduced Ca^{2+} permeability. NR1 and NR3 subunits have binding affinities for glycine, NR2 subunits bind specifically to glutamate [184]. NR1/NR3 receptor is a glycine-responsive channel that does not require L-glutamate binding for activation.

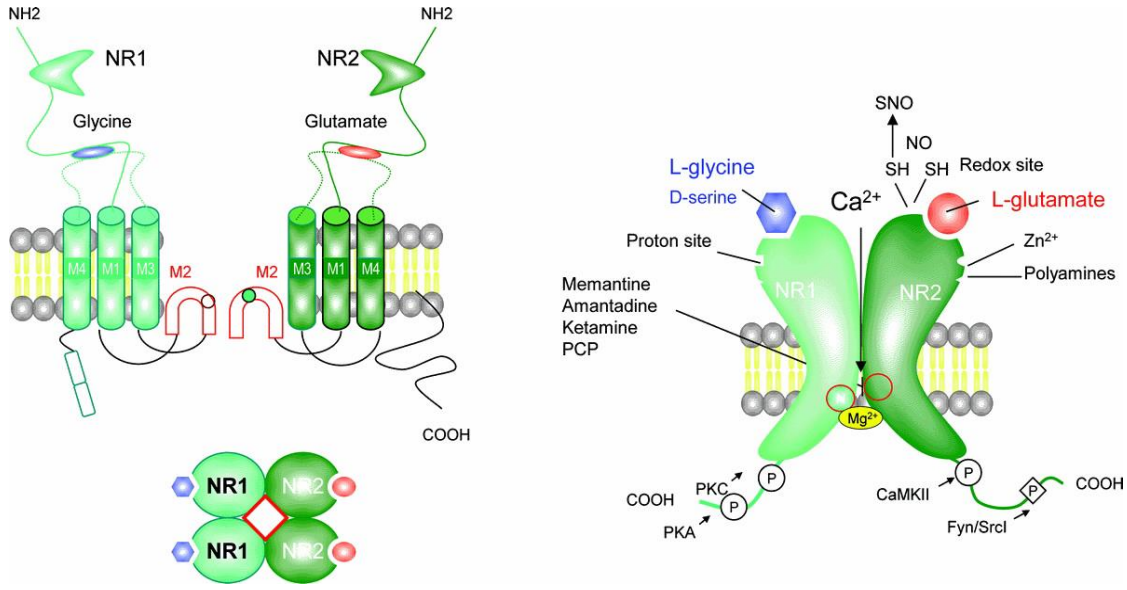

Figure 1.5.2.2: Structure, gating, and allosteric modulation of NMDA receptors (NMDARs)The NMDARs are heteromeric complexes composed of 4 subunits derived from 3 related families: NR1, NR2, and NR3; the NR1 is an obligatory subunit that combines with NR2 or NR3 subunits to form a functional receptor. The typical NMDAR requires 2 NR1 subunits, which bind glycine, and 2 NR2 subunits, which bind glutamate. Each NMDAR subunit contains a large extracellular amino (NH2)-terminal domain (ATD), 3 membrane-spanning domains (M1, M3, M4), a re-entry, or hairpin, loop that forms the pore-lining region (M2), and an intracellular carboxy (COOH)-terminal domain that contains phosphorylation (P) sites. Together, S1 and S2 form a bilobed structure that forms a cavity containing the binding sites for glutamate or glycine. The typical NR1/NR2 receptors are permeable to Ca2+ and their activation requires binding of 2 molecules of glutamate to the NR2 and 2 molecules of glycine (or d-serine) to the NR1 subunit. The pore region contains critical asparagine (N) residues that determine ion selectivity. The NMDAR channel is gated in a voltage-dependent manner by extracellular Mg2+ located in the channel pore. Several modulatory sites affect the function of the NMDAR channels. These include the polyamine site, Zn2+ site, proton-sensitive site, and a redox modulatory site. Nitric oxide (NO) reacts with these critical cysteine residues producing S-nitrosylation (R-SNO). CaMKII = calcium/calmodulin-

dependent kinase II; PKA = protein kinase A; PKC = protein kinase C; Src and Fyn = protein tryosine kinases (Reprinted from Benarroch, Eduardo Neurology 76(20):1750-1757)

NMDA-R subunit assembly, which determines the biological, electrophysiological and pharmacological characteristics of NMDA-R, is a dynamic process where subunit composition is plastic and regulated by developmental stage, neuronal activity and the tissue microenvironment [185, 186]. Some of these are discussed below.

1.5.2 NMDA Receptors: Ontogeny and Tissue Distribution

The distribution and arrangement of NMDA-R subunits is precisely regulated during pre and postnatal development and dependent on the region and surrounding microenvironment. While NR1 subunit expression is ubiquitous throughout the nervous system, both during embryonic stage and adulthood, NR2 subunits demonstrate spatiotemporal changes in their expression pattern [184, 188, 189]. NR2B is the predominant NR2 subunit during early life and its expression declines substantially in the postnatal period. Within 2 weeks after birth, NR2A expression increases rapidly and it replaces NR2B subunit in many regions of the brain. Subunits NR2A and NR2B are abundant inside the forebrain, which is comprised of cortex, striatum, hippocampus and olfactory bulb [184, 188, 189]. Expression of NR2C is restricted to adult cerebellar granule cells and NR2D occurs primarily in the diencephalon and brainstem [184, 188, 189]. Ontogeny of NR3 subunits is unique. Subunit NR3A expression is low in the prenatal period but undergoes significant increase during early postnatal phase. However, NR3A returns to prenatal levels as individual progresses to adulthood. On the contrary, NR3B is

expressed at very low level initially, but increases with age. The presence of NR3B is not only predominant in the motor neurons of brainstem and spinal cord, but also is distributed widely across different regions of CNS [184, 188].

In neurons, NMDA-Rs occur as an integral part of post-synaptic density (PSD), co-localized with a family of scaffolding proteins called membrane-associated guanylate kinases (MAGUKs) [190]. Interestingly, a single post synaptic neuron can express different NR1 isoforms and NR2 isoforms leading to formation of multiple NMDA-R subunit combinations on the same neurons [189, 190]. NMDA-Rs are also described in presynaptic, perisynaptic and extrasynaptic sites thought to be involved in regulating neurotransmitter release, migration and differentiation [190]. Growing literature evidence suggests NMDA-R expression is not CNS exclusive; albeit several peripheral neuronal and non-neuronal sites have physiologically functional NMDA-Rs. Peripherally, NMDA-Rs are localized on the dorsal root ganglions and afferent terminals and regulate transmission of acute pain signals from the visceral organs [191]. Physiological role of NMDA-Rs in other tissues is discussed in another section.

1.5.3 NMDA-R: Agonists, co-agonists and Antagonists

Maximal NMDA-R activation requires simultaneous binding of agonist (glutamate) and co-agonist (glycine or D-Serine) [183, 184, 186]. In comparison to other iGluRs, NMDA-Rs exhibit a very high binding affinity (EC50 ~1µmol L^{-1}) for L-Glutamate [192]. Similarly, engagement of strychnine-insensitive glycine-binding sites on NR1 and NR3 by

glycine is considered crucial for receptor activation [193]. D-Serine, released endogenously by the surrounding astrocytes and glia, is an equally potent co-agonist as glycine [192, 193]. Further, NMDA-R function can be either activated or inhibited by a multitude of allosteric interactions with ions (Zn [184, 192], Pb^{2+} [194]), polyamines [184], and amino acids (L-Aspartate [195] and L-Alanine [196]). Table 1.5.3.1 summarizes various NMDA-R agonists, co-agonists, and allosteric modulators, whose binding sites on the receptor are demonstrated in figure 1.5.2.2.

Table 1.5.3.1 Agonists, co-agonists and allosteric modulators of NMDA Receptors

Agonists	Co-agonists	Allosteric Modulators
L-Glutamate NMDA Quinolinic acid/quinolinate Homocysteine	Glycine D-Serine, L-Serine D-cycloserine	L-Alanine, L-Aspartate, D-Aspartate, Polyamines (spermine and spermidine) Zn^{2+}, Pb^{2+}, Ifenprodil Alcohol, Histamine, Pregnenolone sulfate

Source: Compiled from [184, 188, 192-201]

Polyamines and amino acids (alanine and aspartate) function as positive modulators of NMDA-R activity. By binding to NR2B containing receptors, they augment glycine-binding affinity, thereby increasing the receptor's maximal response. On the contrary, Zn^{2+} inhibits NMDA-R responses in a voltage-dependent manner. NMDA-R activation and function is also influenced by a specific class of neuroactive compounds called

kynurenines, metabolic intermediates generated during tryptophan catabolism catalyzed extrahepatically by indoleamine 2, 3-dioxygenase (IDO, EC 1.13.11.42) [198, 202]. Prominent members include quinolinic acid (QUIN; NMDA-R agonist and neurotoxin), kynurenic acid (KYN; NMDA-R antagonist), 3-hydroxy kynurenic acid, 3-hydroxy anthranilic acid (free radical generator) and picolinic acid (neuroprotective compound) [198]. The role of kynurenines as neuroactive molecules [198] is well established. Also, IDO and kynurenines can even promote Th2 responses and mediate airway inflammation in chronic models of allergic airway disease [203, 204]. While IDO expression is induced in lung DC during lung inflammation [205], serum kynurenine levels are elevated in patients with chronic airway obstruction [206]. Also, previous work from our lab shows that human eosinophils constitutively express IDO [168], which may cause further tryptophan depletion and secretion of immunoactive kynurenines [168]. Kynurenines are involved in regulation of T cell subset polarization towards a Th2 bias by inducing apoptosis in Th1 cells [168], which suggests an important role of these molecules in asthma pathogenesis. Furthermore, proinflammatory cytokines (TNF, IL-6, IL-1β and TGF-β) influence NMDA-R expression and function [207].

Ever since NMDA-Rs were shown to be central mediators of neuropathological disorders, a number of selective and non-selective NMDAR inhibitors/antagonists have been identified, which can be classified as competitive, non-competitive and uncompetitive (summarized in table 1.5.3.2). Competitive NMDAR antagonists are broad spectrum inhibitors that compete with glutamate-binding NR2 subunits. Interestingly, these compounds do exhibit modest subunit specificity, which is high for NR2A and weak for

NR2D (NR2A>NR2B>NR2C>NR2D) [193]. While there have been some interesting discoveries about NMDAR-subunit selective competitive antagonists, none of them showed any promise clinically due to extremely unacceptable side effects and low tolerance exhibited during pre-clinical evaluations [208]. A competitive inhibitor widely used for research purposes is 2-amino-5-phosphonopentanoate ((R)-AP5 or D-AP5), which ideally distinguishes NMDA-R from other iGluRs [193].

Non-competitive antagonists bind to sites independent of NMDA-R agonists and demonstrate a substantially high degree of subtype selectivity, particularly for NR2B subunits. Important among them is phenylethanolamine ifenprodil, whose specificity is ~200-400 fold-higher for NR1/NR2B receptors [193, 208]. Kynurenic acid (KYN) is a naturally occurring non-competitive inhibitor, which binds specifically at the glycine site and is associated with schizophrenia and other neurological disorders originating due to diminished NMDA-R activity [208]. In recent years, glycine-site antagonists are being evaluated extensively considering these drugs have lesser side effects and better therapeutic efficiency for diseases involving NMDA-R hyperactivation and neuronal death via excitotoxicity [208]. Under certain conditions, excessive NMDA-R stimulation in neurons causes increased intracellular Ca^{2+} flux leading to mitochondrial membrane depolarization, permeability transition and activation of intrinsic apoptotic pathways [209]. Uncompetitive inhibitors, require the pore channel to be active and open for binding. Also known as pore blockers, uncompetitive antagonists poorly distinguish between different NMDA-R subtypes. Most prominently known high affinity uncompetitive NMDA-R antagonist is MK-801 or Dizocilpine, which exhibits mild subunit selectivity (<10 fold) for NR2A and

2B subunits [193]. Other examples include ketamine, and phencyclidine (PCP). However, advanced clinical trials using high affinity channel blockers failed as they caused drowsiness and hallucinations in patients [210]. Pre-clinically, low affinity uncompetitive antagonists such as memantine are suggested to be highly effective neuroprotective drugs in neuropathological conditions such as Alzheimer's disease (AD) considering their ability to regulate NMDA-R activity in a controlled manner [210-212]. At present, memantine is the only FDA-approved drug available for the treatment and management of cognitive decline in moderate to severe AD patients [210]. A recent multicentric randomized placebo-controlled double-blind clinical study done by Grossberg *et al* showed that administration of single dose of extended-release memantine (28mg once daily) to AD patients' tremendously improved their cognitive functions such as behavior and speech [211]. Similarly, Hellweg *et al*, using post hoc responder analyses method showed that memantine significantly delayed clinical worsening of AD patients with minimum side-effects, compared to placebo controls [213]. However, it is important to note that glutamatergic signaling is severely degenerated during late stages of AD, along with significantly decreased number of neuronal NMDA-Rs [214]. In this regard, effects of memantine on NMDA-R are debatable and require further clarification. Some other uncompetitive inhibitors currently in clinic include amantadine (in Parkinson's disease), dextromethorphan (anti-tussive agent in cough medicines) and ketamine (used as a narcotic and anesthetic agent) [208]. Also, ketamine is administered as a bronchodilator in patients suffering with "status asthmaticus" or fatal asthma [215], which suggests pathological importance of NMDA-Rs in respiratory diseases.

Table 1.5.3.2: Competitive, non-competitive and uncompetitive NMDA-R antagonists

Competitive	Non-competitive	Uncompetitive
(R)-AP5	Ifenprodil	Mg^{2+}
(R)-AP7	Haloperidol	MK-801 (Dizocilpine)
5,7-dichlorokynurenic acid	Kynurenic acid	Ketamine
(R)-CPP		Memantine
PPDA		Phencyclidine (PCP)
PEAQX		Dextromethorphan
7-chlorokynurenic acid		Amantadine
Selfotel		Argiotensin-636

Source: Adapted from [183, 193, 208]

1.5.4 NMDA-Rs in Health and Disease

Considered one of the most important neurotransmitter receptors in the brain, NMDA-Rs regulate a myriad of physiological functions, both in neuronal and non-non-neuronal tissues. Fundamentally, NMDA-Rs convert neuronal stimuli into biochemical signals and transmit them as functional responses across neurons. A key property that differentiates NMDA-Rs from AMPA-Rs and kainate receptors is their ability to demonstrate a longer channel conductance owing to their slower deactivation kinetics [184]. Listed below are some other unique properties exhibited by NMDA-R in comparison to other iGluRs.

a. *NMDA-R activation/deactivation is a voltage-gated process exclusively dependent on the presence of magnesium (Mg^{2+}) ion as channel blocker at resting membrane potential* [186]. However, receptors containing NR3B subunit seems to be an exception to this rule as these channels are presumed to be Mg^{2+}-insensitive. Similarly, complexes containing NR2C and/or NR2D subunits display lower Mg^{2+} sensitivity [184].

b. *NMDA-R exhibit relatively higher permeability to Ca^{2+} than AMPA and Kainate receptors.* However, Ca^{2+} conductance levels are again dependent on the assembled subunits on the membrane, partly regulated by agonist sensitivity. For instance, receptors containing NR2A demonstrate high Ca^{2+} conductance and faster deactivation, but lesser affinity to glutamate. On the contrary, NR2D containing complexes display slower deactivation, stronger glutamate binding and sustained Ca^{2+} permeability [184]

The human brain is capable of preserving countless transient everyday life experiences as everlasting memories and respond accordingly, which is attributed to dynamic changes in the strength of neuronal communication at the synaptic junctions, also known as synaptic plasticity [216]. Available literature strongly suggests regulated calcium entry through AMPA-R, followed by NMDA-R activation and subsequent stimulation of Ca^{2+}-dependent intracellular signaling as central events for synaptic plasticity, which involves learning and memory processes mediated via LTP and LTD [216].

LTP is defined as “long-lasting increase in synaptic strength” and efficacy following a high-frequency excitatory potential. On the contrary, LTD is explained as “persistent weakening of synaptic transmission” occurring due to low-frequency stimulation of nerve terminals [217]. It is interesting to note that LTP and LTD are not exhibited by the same synapse in normal circumstances. LTP was first described in the dentate gyrus of hippocampus as a potential mechanism for synaptic plasticity by Terje Lomo in 1966 [218, 219]. Later, seminal observations by Collingridge *et al* and others identified and confirmed NMDA-R activation as a quintessential phenomenon underlying LTP [220-222]. Long term potentiation occurs as a consequence of prolonged post-synaptic membrane depolarization by high frequency action-potential due to substantial activation of AMPA-R. At resting membrane potential, Mg^{2+} ions bind strongly to NMDA-R channel pore and block receptor activation. During LTP, copious amounts of agonist (glutamate) and co-agonist (glycine) are released from the pre-synaptic terminals following a strong excitatory synaptic potential. Binding of glutamate and glycine to AMPA-Rs leads to membrane depolarization at the post-synaptic terminals via influx of Na^{+} ions, eventually expelling Mg^{2+} ions from the NMDA-R core. As a result, channels are opened allowing flux of Ca^{2+} and Na^{+} ions into the cells and efflux of K^{+} ions to the outside [186, 200, 223]. Prolonged and substantial increase in transient $[Ca^{2+}]_i$ activates various downstream Ca^{2+}-responsive proteins such as protein kinase A (PKA) and CaM/CaMK-II (calmodulin/calmodulin-dependent protein kinase II complex) [224], which in turn trigger multiple signaling cascades and gene transcription. An interesting mechanism underlying LTP is membrane recruitment of functional AMPA-Rs and transient perturbation in surface NMDA-R: AMPA-R ratio [225, 226]. This involves regulated trafficking of

GluA1/GluA2-containing AMPA-Rs and NR2A/NR2B-containing NMDA-Rs towards the post-synaptic membrane for sustained period of time, thus strengthening of excitatory synapses. Available evidence suggests enhanced membrane insertion of AMPA-Rs during LTP occurs as a result of activation of CAM/CAMK-II complex, which promotes increased phosphorylation of AMPA-Rs by PKA and PKC. This leads to increased receptor exocytosis towards to postsynaptic densities (PSD), which occurs at a rapid pace following membrane depolarization. On the contrary, insertion of NMDA-Rs occurs at a relatively slower rate following AMPA-R trafficking [223, 227-229]. Conversely to LTP, LTD involves low frequency action potentials between nerve synapses that cause lesser glutamate release from presynaptic terminals. Depleted neurotransmitter levels cause inadequate AMPA-R activation and membrane depolarization, which culminates into insufficient opening of NMDA-R channels. As a result, modest elevations in cytoplasmic Ca^{2+} levels occur for a long duration, thereby weakening of synaptic communication between neurons [223, 228]. It is suggested that insufficient calcium elevations during LTD activates calcium/calmodulin-dependent protein phosphatases like calcineurin and PP1, causing increased dephosphorylation of AMPA-Rs and enhanced receptor internalization via endocytosis [230, 231]. Due to this, AMPA-R levels on the membrane decrease favoring diminished synaptic strength [223, 228, 231]. Taken together, it is now well-established that dynamic regulation and activity-based exocytosis/endocytosis of AMPA-R is crucial for NMDA-R-mediated synaptic transmission. However, we still do not quite understand the underlying signaling mechanisms that are activated to maintain LTP/LTD.

NMDA-R also functions as a co-incident sensor in neurons and participates in synapse maturation, dendrite growth and neuronal co-ordination during development and adulthood [224]. Defect in this process causes severe mental retardation in young children. Using various antagonists and animal models, researchers have also identified NMDA-R role in verbal fluency, speech recognition, spatial learning, object recognition, neuronal survival and cognition. Developmental changes in NMDA-R subunit patterns are critical for the onset of "ocular dominance plasticity" inside cortical neurons [189, 224, 232]. NMDA-Rs expressed on inhibitory neuronal circuits induce release of inhibitory neurotransmitter GABA and regulate GABA-receptor functioning, which suggests physiological role of NMDA-R is much elaborate than anticipated [233].

Deregulated glutamatergic neurotransmission as a result of dysfunctional NMDA-R, either through hyperactivation or hypoactivation, leads to cellular damage. NMDA-R hyperactivation either due to presence of excess glutamate in the synapse or potentiation of receptor activity leads to unabated Ca^{2+} influx and triggers activation of cellular nucleases, phospholipases and disruption of mitochondrial membrane potential [234]. These events lead to activation of mitochondrial caspases, increased oxidative stress, release of pro-apoptotic proteins and DNA damage, which culminate as neuronal cell death [234]. Selective and sustained cellular "excitotoxicity" is widely implicated in the progression of HIV-associated dementia, Alzheimer's disease, stroke, trauma, ischemia, multiple sclerosis, epilepsy and ALS [224, 234]. Interestingly, excitotoxicity responses seem to be dependent on NMDA-R subunit and the receptor location; albeit, these issues have no agreed consensus [190, 224]. NMDA-R hypoactivation is a key pathological

feature in schizophrenia and aging [190, 235]. Reduced NMDA-R activity interferes with the excitatory and inhibitory neuronal circuits, thereby causing impaired cognitive abilities and neuropsychosis in schizophrenics [235]. Some plausible explanations for this phenomenon include decreased NR1 and NR2B expression causing rearrangement of receptor subunit configuration, reduced agonist binding sites on NMDA-Rs and changes in agonist affinity [235]. However, these hypotheses still remain under investigation. While NMDA-Rs are essential for pain sensitization by the nervous system, auto-antibodies against NMDA-Rs mediate the pathogenesis of autoimmune NMDA receptor encephalitis, a treatable form of autoimmune disorder affecting CNS of young children [234].

Emerging evidence suggests an essential role of glutamatergic signaling in maintaining normal physiology of various peripheral tissues. Amongst the iGluRs described till date, only NMDA-Rs are suggested to be widely distributed among peripheral tissues suggesting these receptors have a much broader significance beyond the nervous system. NMDA-R are expressed on non-neuronal tissues, including bone [236], skin [237], kidneys [238], heart [239], pancreas [240], gastrointestinal tract [241], parathyroid gland [242], vascular endothelium [243], lungs [244], airways [244] and cells of the immune system [245, 246]. In bones, NMDA-Rs expressed on osteoblasts, and osteoclasts regulate bone remodeling and Ca^{2+} assimilation in the tissues [236, 247, 248]. While NMDA-Rs regulate parathyroid hormone release in a MAPK-dependent manner [242], receptors expressed on renal podocytes are suggested in the maintenance of glomerular filtration rate and proximal reabsorption [238, 249]. In the skin, functional NMDA-Rs participate in keratinocyte re-epithelialization, cutaneous barrier formation and wound repair [237].

NMDA-Rs are also linked to regulation of insulin release from pancreas, intestinal smooth muscle contractions, modulation of gastric secretions, regulation of innate and adaptive immune responses by T-lymphocytes and release of catecholamines from adrenal glands [173, 250, 251]. While these observations elucidate a crucial interplay between neuronal and non-neuronal systems, we still require additional investigations to validate these findings as possible future therapeutic targets. Functional NMDA-Rs were described by some research groups in lungs and airways; their physiological and pathological significance is described in the next section.

1.6.0 NMDA RECEPTORS IN LUNGS AND AIRWAYS

To date, the role of the glutamatergic system, particularly NMDA-Rs, in regulating airway reactivity in the lungs has remained poorly understood. Pioneering studies from Dr. Sami I. Said and his group, using *in vivo* animal models for lung inflammation, have shown that activated NMDA-Rs might play a key role in lung health, inflammation and airway pathology [79, 244, 252]. They observed that perfusion of rat lungs with NMDA can induce acute lung injury and pulmonary edema, which was prevented by nitric oxide synthase inhibitors [49, 79, 252]. Similarly, non-selective NMDA-R antagonist MK-801 significantly attenuated capsaicin-induced airway responses in guinea-pig lungs. Further, studies done by Dickman *et al* and others demonstrated perfused rat lungs, trachea and alveolar macrophages expressed different NMDA-R subunits, including the obligatory NR1, and that the receptor activation could interfere with synthesis of pulmonary surfactants [244, 253]. Also, there is evidence that human lung cells express different

NMDA-R subunits, at least in various cancer cell lines [254, 255]. Said *et al* also showed that perfusion of guinea pig tracheal segments with NMDA increased resting muscle tone and enhanced airway contractility in response to acetylcholine, suggesting that NMDA might induce AHR and bronchial constriction [79, 252], two characteristic pathological symptoms observed in asthma. These findings were substantiated, albeit indirectly by Strapkova *et al* and others, where NMDA administration considerably augmented airway responsiveness to ACh in an ovalbumin-induced allergic asthma model, which were blunted using receptor-specific antagonists [256].

Considering ASM cells are principal contributors to normal airway functioning and pathogenesis, NMDA-R expression and its physiological function on HASM cells is yet to be elucidated. My project investigates the expression of multiple NMDA-R subunits in HASM cells, defines their functional phenotype and identifies receptor significance in normal vs. inflamed murine airways.

CHAPTER 2

2.1.0 STUDY RATIONALE

- The ASM layer surrounding the hollow airways in the lung is indispensable for normal pulmonary function. While it protects the structure of human airways, it stabilizes airways from collapsing in response to dynamic changes happening in airway caliber and stiffness. In addition, a properly structured ASM layer is essential for optimal ventilation and gas exchange in the lungs, promoting uniform lymphatic and venous flow, assisting in propulsion of mucus layer and protecting peripheral lungs from environmental insults [4].
- Multifunctional ability of ASM cells is attributed to their pleiotropic nature and functional plasticity. While ASM cells are classically associated with airway contraction and relaxation, these cells possess an intrinsic ability to proliferate, migrate and secrete multiple cytokines, chemokines and ECM proteins; and exist as heterogeneous sub-populations [257, 258].
- Dynamic regulation of cellular calcium mobilization is an essential contributor to ASM structure and function, both in health and disease. Under resting or basal conditions, ASM cells display low free cytosolic calcium levels (~100-200nmol/L). Upon activation by agonists, substantial influx in $[Ca^{2+}]_i$ is facilitated by various ROCCs and SOCCs. However, cellular mechanisms underlying ASM Ca^{2+} handling and sensitivity are complicated and remain poorly understood in all circumstances.

- ASM cells, in response to prolonged exposure to multiple inflammatory mediators, demonstrate altered calcium homeostasis and dysfunctional phenotypic plasticity, which are considered key events underlying AHR. Alternatively, emerging research suggests ASM-driven AHR precedes airway inflammation and triggers disease progression upon provocation by allergens, viruses and/or environmental irritants. An important mechanism underlying AHR is hyper-excitatory condition of the airways, primarily driven via activation of cellular pathways of excitatory amino acids such as glutamate in the lungs [259].
- Glutamatergic signaling is mediated through activation of various glutamate receptors. Important among those are NMDA-Rs, a unique family of glutamate ion channels widely recognized for their high calcium permeability, apart from sodium and potassium ions.
- *In vitro* observations using animal tissues suggest neuronal NMDA-Rs are expressed in various compartments of the lung. Also, NMDA-R activation induced AHR in experimental animal models of airway inflammation, which was successfully alleviated using specific receptor antagonists.

Given the presence of various NMDA-R agonists, including glutamate and NMDA, contributing to airway inflammation and hyperreactivity, I set an objective to examine the novel presence and regulation of functional NMDA-R in human ASM cells. Further, I aimed to investigate their contribution towards regulating normal and asthmatic airway responses.

2.1.1 GLOBAL HYPOTHESIS

Glutamate exerts biological effects in human ASM cells through NMDA-R and contributes to airway smooth muscle contraction and airway responsiveness

2.1.2 OVERARCHING GOAL AND SPECIFIC AIMS

The overarching goal of this study is to elucidate NMDA-R expression, Ca^{2+} mobilization and molecular regulation in HASM cells and elaborate receptor's physiological and pathological significance. To test my overall hypothesis, I will examine three major objectives:

Objective 1: *Determine the expression of functional NMDA-R on HASM cells, in vitro and ex vivo.*

Detailed explanation of study rationale for this objective has been outlined in the introductory section of Chapter 4. We hypothesized that ***NMDA-R are expressed on ASM and mediate functional changes in these cells upon stimulation***.

Using cultured human ASM cells, I examined the subunit composition of NMDA-Rs, and their ability to mobilize calcium intracellularly upon activation. Further, I also

examined the affect on NMDA-R activation on HASM contraction, using both in vitro and ex vivo methodologies.

Objective 2: *Identify the effect of airway microenvironment on NMDA-R expression and function in HASM cells, in vitro and ex vivo.*

Study rationale has been explained in Chapter 5. I hypothesized that *the* ***local airway milieu modulates NMDA-R subunit expression, potentiates NMDA-R mediated*** $[Ca^{2+}]_i$ ***influxes, and contributes to contractile responses in HASM cells.***

To test this hypothesis, I used cultured HASM cells to describe TNF's involvement in the regulation of NMDA-R expression and receptor-mediated calcium inflow. Further, I also demonstrated TNF's unique role in re-defining NMDA-R-induced airway responses using cultured murine explants. I further characterized the effect of inflammatory microenvironment on NMDA-R function using a HDM-induced mouse model of allergic asthma.

3.0 CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1.0. ETHICS STATEMENT & ANIMALS

All the experiments were approved by Human Research Ethics Board of University of Manitoba, Winnipeg, Manitoba, Canada. Written and informed consent was obtained from all patients for ASM harvesting.

Female Balb/c (8-10 weeks old) and C57BL/6 (8-10 weeks old) were purchased from Central Animal Care Services, University of Manitoba, Winnipeg, Canada. Animals were allowed to acclimatize for a week before any procedures were done and maintained on a regular laboratory chow diet. All the procedures were performed according to established standard operating protocols. Experimental protocols requiring animal handling and usage were approved by the Animal Ethics Committee of the University of Manitoba, Winnipeg, Manitoba, Canada.

3.2.0 REAGENTS

3.2.1 Chemicals

Recombinant human IFNγ, IL-4 and TNF were purchased from Peprotech Inc, USA. Fetal bovine serum (FBS) was purchased from PAA Laboratories GmBH (Austria). Dulbecco's modified Eagle's medium (DMEM), Ham's F12K, Penicillin-Streptomycin solution, trypsin-EDTA, insulin-transferrin-selenium (ITS) mix and antibiotic-antimycotic

solution were purchased from Lonza Inc (USA). *N*-methyl-*D*-aspartate (NMDA) and acetyl-β-methacholine chloride were obtained from Sigma-Aldrich Inc (Oakville, ON).

3.2.2 Antibodies

Polyclonal anti-NR1 antibody (sc-1467) and polyclonal goat anti-IgG isotype control antibody were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Monoclonal anti-β-actin antibody was purchased from Sigma-Aldrich Inc (Oakville, ON).

3.2.3 Inhibitors

D-(-)-2-amino-5-phosphopentanoic acid (D-AP5; CAS-79055-68-8) was purchased from Tocris Bioscience (UK). Cyclooxygenase inhibitor indomethacin and nitric oxide synthase inhibitor L-NAME (L-N^G-nitroarginine methyl ester) were purchased from Sigma-Aldrich Inc (Oakville, ON).

3.3.0 CULTURE OF HUMAN AIRWAY SMOOTH MUSCLE CELLS

For our study, we used primary human airway smooth muscle (HASM) cells and hTERT-immortalized HASM cells. For the sake of convenience, these cells will be collectively referred in my thesis as human airway smooth muscle cells (HASM or HASMC or ASMC or ASM cells). HASMCs were obtained from macroscopically healthy 2^{nd}-4^{th} generation mainstem bronchi removed from patients undergoing lung resection surgery (in collaboration with Dr. Helmut Unruh, Head of the Section of Thoracic Surgery,

University of Manitoba). Isolation of single myocyte populations was done as per the procedure described by Halayko *et al* [258]. These cells classified as "primary ASM cells" were used between passages 2-4. Immortalization of primary ASM cells was accomplished by infecting cells with a retrovirus vector carrying human telomerase reverse transcriptase (hTERT) gene. Protocol for stable expression of hTERT gene was done as per the method outlined by Gosens *et al* [92]. Stable expression of hTERT gene in low-passage and primary cultures of ASM cells extends their lifespan without allowing them to undergo senescence [92]. These cells retain their ability to express contractile proteins and phenotypic capability to exhibit maximum response to contractile agonists such as methacholine, bradykinin and histamine. For all experiments, hTERT cells were used at passages 20-23.

3.3.1 Cell Culture and Stimulations

For all *in vitro* experiments, HASM cells were cultured in complete DMEM supplemented with 10% FBS, 100µg/ml streptomycin and 100U/ml penicillin. Cells were cultured at 37°C with 5% CO_2. Unless otherwise mentioned, cells were grown on uncoated plastic surface to a sub-confluent stage (70-80%). Cells were dissociated using sterile 1X PBS/2mM EDTA solution and seeded in a 24-well tissue culture dish (Nunc, Thermo Fisher Scientific Inc, US) @ 3 x 10^4 cells per well. Cells were serum-deprived for 48h to synchronize their growth at G_0/G_1 stage. For serum-deprivation, HAM's F12K medium supplemented with 1X ITS (5µg/ml insulin, 5µg/ml transferring and 5ng/ml selenium) was used. For stimulation, fresh HAM's F12K + ITS medium containing human recombinant

IL-4, IFN-γ or TNF were used for stipulated time periods as per the specific needs of the experiment.

3.4.0 RNA ISOLATION, QPCR AND RT-PCR

3.4.1 RNA Isolation and cDNA Synthesis

Total cellular RNA from 48h serum-deprived HASM cells was isolated using E.Z.N.A Total RNA Isolation Kit (Omega Bio-tek), as per the manufacturer's instructions. RNA was quantified by measuring absorbance at 260nm and 280nm using Nanodrop (Thermo Fisher Scientific Inc, US). Reverse transcription for first-strand cDNA synthesis was done with qScript^{TM} cDNA supermix (Quanta Biosciences, MD) containing equimolar concentrations of oligodTs and random hexamers and using 200ng DNase-treated total RNA as per the protocol mentioned by the manufacturer.

3.4.2 QPCR (Real-time RT-PCR)

The mRNA expression level for NMDA-R subunits in HASM cells was determined using PerfeCTa SYBR® Green SuperMix (Quanta Biosciences, MD) and Roche LightCycler 480 qPCR system. The sequence for oligonucleotide primers used and their product sizes are summarized in table 3.4.3. Subunit-specific primers were designed across intron/exon boundaries using IDT primer quest software (www.idt.com) while specificity was determined using NCBI Primer Blast and subsequent melt curve analysis (Roche LightCycler 480). We used 18s rRNA detection as a reference standard for normalization.

Absolute quantification of mRNA for NMDA-R subunits was determined by standard curve method, where standards for all the target genes were created from Human Universal Reference (HUR) total RNA (Clontech Laboratories Inc, CA). First-strand cDNA synthesis was done using Platinum cDNA supermix using subunit-specific primers as listed in table 3.4.3. PCR products were isolated from 2% w/v agarose gel using Qiagen II Agarose gel extraction kit (Qiagen Inc, ON). The amount of extracted cDNA was quantified by spectrophotometry using Nanodrop and expressed as copy number. To quantify the exact copy number of each target gene in test samples, a standard curve was generated using a 7-point 10-fold dilution series over 6 orders of magnitude (1×10^7 to 1×10^1) using quantified concentrations of the standards. Standard curves with a slope of 3.6 $\pm$ 0.3 and efficiency range of 1.8 – 2.0 (equivalent to 90-100% amplification efficiency) were considered for analysis. Aliquots of cDNA equivalent to 5ng of total RNA samples were used for each real-time PCR reaction. The mRNA expression levels were normalized to 18S rRNA levels for each sample. Data was represented as copy number/ng RNA normalized to 18s. Product specificity was determined by melting curve analysis. The mRNA isolated from SK-N-SH cells was used as a positive control [260].

3.4.3 RT-PCR

To determine NR1 expression in mouse lungs and brain, frozen tissues were homogenized and total RNA was isolated using RNeasy Plus Mini Kit (Qiagen Inc, ON). First strand cDNA was synthesized using 1µg RNA as template. Gene of interest was amplified in a 25µL reaction (as per manufacturer's instructions) using EconoTaq PLUS

GREEN 2X master mix (Lucigen) using primers specifically designed for mouse NR subunits (no. of PCR cycles = 35; primer sequence and amplicon size is indicated in Table 3.4.3). TATA-box binding protein (TBP) was used as an internal standard.

3.5.0 WESTERN BLOTTING, IMMUNOFLUORESCENCE AND FLOW CYTOMETRY

3.5.1 Western blotting

To determine NR1 subunit expression, HASM cell lysates were prepared using a commercially available TPE kit (Total Protein Extraction Kit, GBiosciences Inc, US) as per manufacturer's instructions. Lysis buffer was supplemented with 1X PMSF and 1X protease inhibitor cocktail (SIGMAFAST^{TM}, Sigma-Aldrich, St.Louis, MO, US). Protein concentration was determined by DC^{TM} protein assay kit (Bio-Rad Inc). For immunoblotting, samples were separated using a 10% sodium dodecyl sulphate - polyacrylamide gel (SDS-PAGE) and transferred on to a PVDF membrane. Blots were blocked in blocking buffer (5% non-fat powdered milk diluted in Tris-buffered saline containing 0.1% Tween-20) for 1h at room temperature. Next, membranes were incubated at 4°C overnight with anti-NR1 antibody (1:250, Santacruz Biotechnology Inc) diluted in blocking buffer. After 3 washes (10min each) with 1X Tris-buffered saline-Tween 20 (TBST) buffer, membranes were incubated with HRP-conjugated secondary anti-goat IgG (1:15,000 dilution in blocking buffer) for 60min at room temperature. Blots were washed 6 times (10 min each) with 1X-TBST buffer and developed by Clarity^{TM} Western enhanced chemiluminescence substrate (Bio-Rad Laboratories, CA).

3.5.2 Immunofluorescence

To visualize NR1 expression by immunofluorescence, HASM cells were cultured in 4-well Lab-Tek II chamber slides (Nunc) for 96h. Cells were washed (5min each) with 1X cytoskeleton buffer (CSB; 10mM 4-Morpholineethanesulfonic acid, 150mM NaCl, 5mM $MgCl_2$, 0.9mg/ml glucose, 5mM EGTA, pH 7.0) and fixed for 15min at room temperature using 4% paraformaldehyde (PFA) prepared in 1X CSB. Again, cells were washed 3 times (5min each) with 1X CSB and blocked using 0.5% Gelatin dissolved in 1X phosphate-buffered saline (PBS) for 60min at room temperature. After washing cells with 1X PBS (3 x 5min), anti-NR1 (BD, diluted 1:100in 0.1% Gelatin in PBS) antibody was added and incubated overnight at 4°C. Isotype-matched mIgG antibody was used as negative control. Later, cells were washed with 1X PBS (3 x 5min) and incubated with Biotinylated-Donkey anti-mouse IgG (H+L) secondary antibody (Jackson Laboratories) for 60min at room temperature. Cells were washed with 1X PBS (3 X 5min) and incubated with AlexaFluor 488-conjugated streptavidin (Invitrogen) in dark for 1h at room temperature. Later, cells were washed (3 x 5min; 1X PBS) and coverslips were mounted with ProLong Gold Antifade medium containing DAPI (Molecular Probes). DAPI was used to stain the nuclei. Fluorescent images were captured using an Olympus BX-51 fluorescent confocal microscope.

3.5.3 Flow Cytometry

For intracellular staining, HASM cells were detached using ice-cold PBS containing 2mM EDTA, washed with PBS and fixed with ice-cold 2% PFA in PBS for

10min. After washing with flow buffer (1% BSA in PBS), cells were permeabilized using 0.1% saponin in flow buffer for 10min at 4°C. After washing cells with flow buffer (2 x 5min), cells were incubated with primary antibodies (anti-NR1; 1:100; goat polyclonal; Santa Cruz Biotechnology) for 1h at 4°C. Cells washed three times with flow buffer, and labeled with AlexaFluor-488 conjugated Donkey anti-goat IgG (H+L) secondary antibody (1:350; Life Technologies) for 1h at 4°C. Goat IgG was used as isotype control. Fluorescence intensity was detected by BD FACS Canto II and data was analyzed by FlowJo software (BD Biosciences, San Jose, CA).

3.6.0 $[Ca^{2+}]_i$ FLUX MEASUREMENTS

Real-time quantification of intracellular cytosolic Ca^{2+} ($[Ca2+]_i$) was performed using Fura-2AM (Invitrogen, NY), a Ca^{2+}-sensitive ratiometric fluorescent dye [261]. For measurement, cells were grown on Lab-tek coverglass chamber slides (Nunc), washed with HBSS/HEPES buffer containing 0.1% BSA (HHB) and incubated with 5 μg/ml Fura-2 AM (room temperature, 1 hour) in HHB containing 0.01% pluronic acid. After washing, cells were further incubated in HHB (room temperature, 1 hour) to facilitate Fura-2AM de-esterification. Real-time changes in $[Ca^{2+}]_i$ were recorded using an Olympus LX-70 inverted epifluorescent microscope (20× objective) coupled to a Nikon CCD camera controlled by NIS imaging software (NIKON Instruments Inc., NY, US). The system was further coupled to a Sutter Instruments Lambda 10-2 filter wheel and controller with repeated 100ms excitation at 340 and 380 nm; emission at 510 nm was recorded continuously for up to 5 minutes after the addition of contractile agonists. The ratio of

emission at 510 nm excited by 340- and 380-nm light was converted to $[Ca^{2+}]_i$ values from a calibration curve generated using Ca^{2+} standards and calculated by the method of Grynkiewicz [262].

3.7.0 2D-COLLAGEN GEL CONTRACTION ASSAYS

3.7.1 Preparation of collagen gel lattices

Type I collagen gel assay was performed as per the protocol outlined by Takayama et al [263]. Briefly, 7mL of 3mg/mL cold PureCol collagen solution (Advanced Biomatrix, CA, US) was diluted in 2mL of sterile 5X FBS-free DMEM and pH was adjusted to 7.4 using 1N NaOH. Final volume was made up to 10mL using sterile dH_2O. 500µL of this solution was aliquoted in each well of a 24-well nunc tissue culture dish and allowed to polymerize at 37°C for 1h. HASM cells were layered on top of the polymerized collagen gels at $0.5x10^5$ cells/mL in DMEM supplemented with 10% FBS and 1X penicillin-streptomycin solution. Cells were allowed to grow in an incubator at 37°C and 5%CO_2. 24h later, gel lattices layered with HASM cells were washed gently with 1X HBSS. Complete growth medium was replaced with Ham's F12K supplemented with 1X ITS to initiate serum deprivation of cells. Following 48h serum-deprivation, cells were stimulated with fresh Ham's F12K + ITS medium containing IL-4, IFN-γ or TNF for another 48h.

3.7.2 Measurement of Collagen Gel Contraction

To determine the effect of NMDA-R agonists on contractility of HASM cells, edges of the collagen gel lattices were released from wells and allowed to contract spontaneously

for 2h (t=0), after which respective agonists were added. Images were captured at regular time intervals using a Canon digital camera and analyzed using ImageJ software (NCBI). Data was represented as % contraction, which was calculated by subtracting area of collagen gel at a particular time point from gel area at t=0 divided by 100. Percentage decrease in gel area represents the contraction ability of HASM cells in response to different conditions. Contractile response to bradykinin was used as a positive control.

3.8.0 MEASURING AIRWAY CONTRACTION USING MURINE THIN-CUT LUNG SLICE (TCLS) SYSTEM

3.8.1 Preparation of TCLS

Protocol described by Perez-Zoghbi *et al* [140] was used for the preparation of TCLS. Briefly, 8-10wk old female Balb/c or C57BL/6 mice were anesthesized by intra-peritoneal injection of 0.1mL of sodium pentobarbital (90mg/kg body weight). After tracheal cannulation, lungs were inflated with ~1.3mL of warm 2% low-melting agarose in 1X HBSS followed by injecting ~0.2mL of air to flush the agarose to the distal alveoli. The lungs were cooled at 4°C to allow agarose polymerization. After opening the thoracic cavity, left and right lobes were removed and sectioned using a vibratome (model EMS-4000; Electron Microscope Sciences, EMS) into slices of ~180 µm thickness starting at the lung periphery. Temperature was maintained between 4-5°C and ice-cold HBSS was used during the process. Slices were maintained (37°C and 5% CO_2) in DMEM supplemented with 1X antibiotic-antimycotic solution (Lonza Inc) and medium was changed every 24h.

3.8.2 Measurement of airway contraction

Airway slices maintained between 24-72h were used for our experiments. For measuring airway responses, a large cover glass was placed on a plexiglass support and a perfusion chamber was custom-made on the coverglass using silicone (Dow). Lung slices were held in place inside the chamber with the help of a small nylon mesh. A hole was made in the middle of nylon mesh to allow microscopic observation of selected airway. A constant perfusion rate was maintained within the chamber using a gravity-fed perfusion chamber connected to a multi-tube manifold, which was controlled by a valve-system. Phase-contrast images of airway narrowing were recorded using an inverted microscope with a 10X objective connected to a CCD camera. VideoSavant 4.0 software (IO Industries Inc) was used for image acquisition and digital recording of images in a time-lapse mode. Recordings were analyzed with "NIH /Scion Image" software (Scion Corp.). The airway lumen area was determined by adding the number of pixels below a selected threshold grey level with respect to time. All experiments were performed at room temperature (RT).

3.9.0 ISOMETRIC FORCE MEASUREMENT IN MOUSE TRACHEA

3.9.1 Preparation, denudation and culture of isolated murine tracheal rings

Measurement of isometric force in murine tracheal rings was done as per the protocol described by Sharma *et al* [264]. Briefly, trachea along with entire respiratory tract and heart was removed from 8-10wk old female Balb/c mice anesthesized using sodium pentobarbital injection (90mg/kg body weight) and placed in ice-cold Krebs-Henseleit bicarbonate solution (K-H; composition in mM –118 NaCl, 23.5 $NaCO_3$, 4.69 KCl, 1.18

KH_2PO_4, 1.0 $MgCl_2$, 2.5 $CaCl_2$ and 5.55 dextrose). The K-H bicarbonate solution was gassed with 95% O_2- 5% CO_2 mixture to maintain a pH between 7.3 and 7.5.

Tracheal isolation was done in a dissecting dish using ice-cold K-H solution. The apex of the heart and the voice box of the trachea were pinned to the dish. Lungs were removed and preserved for RNA and protein isolation. Carefully, extraneous tissue surrounding the trachea was removed under a microscope. Each isolated trachea was cut into 4 rings, with each ring containing 3-4 cartilages. Tracheal rings were stored in K-H bicarbonate buffer before mounting on to a DMT force transducer system (Danish Myo Technology, Aarhus, Denmark). For denudation of epithelium, tracheal rings were threaded on to silk surgical strings, and rolled gently for 3 times on a wet tissue paper soaked with K-H solution.

3.9.2 Force equilibration in isolated tracheal rings

Tracheal rings were randomly selected to be placed in each well of a force transducer filled with 5mL of K-H bicarbonate solution with a constant gas flow comprised of 95% O_2 and 5% CO_2. Temperature in the transducer was maintained at 37°C. Each ring was mounted in one well of the transducer and positioned in such a way that the membranous side of the trachea faced upwards to ensure consistency.

To obtain a steady baseline measurement, all the tracheal rings were initially stretched in such a way to obtain ~0.6mN force and allowed to relax for 30 minutes, with intermittent K-H buffer changes. During this period, the baseline was frequently adjusted to 0.6mN. This procedure was done to ensure optimal resting tension and tracheal rings are

stable enough to generate maximal force over a constant length. To establish reference length or maximal contractile force generated by a tracheal ring due to membrane depolarization, the rings were treated with 3 exposures of 66mM KCl-substituted K-H (also known as high K^+ solution). For this, 5mL of K-H bicarbonate solution present in the well was replaced with 5mL high K^+ buffer (composition in mM – 59 NaCl, 23.5 NaH_2CO3, 66KCl, 1.18 KH_2PO_4, 1$MgCl_2$, 2.5 $CaCl_2$ and 5.55 dextrose) and force measurements were recorded for 2min. Between each high K^+ treatment, tracheal rings were washed twice with K-H solution for 20min, while resting force was adjusted to 0.6mN to re-establish the baseline. Isometric contractile force developed for each tracheal ring preparation was determined by calculating the difference between the baseline reading and the peak plateau reached by the trachea before and after 3rd high K^+ treatment respectively. This difference was used as the reference force for subsequent force measurements or contractile responses elicited due to stimulation with respective agonists.

3.9.3 NMDA-concentration responses studies

To determine the effect of NMDA-R activation on the isometric force generated by trachea, NMDA cumulative dose-response studies were performed (15μM – 3mM) on epithelium intact and denuded rings. For each dose, recordings were obtained for at least 2 min. The concentration of NMDA was increased 2 fold until the accumulated agonist concentration was 3mM. Force generated due to 10^{-5}M MCh was used as a positive control and to determine the maximal response to a contractile agonist. To determine the effect of TNF on NMDA-induced isometric force, murine tracheal rings were isolated from female Balb/c

mice and cultured in FBS-free DMEM supplemented with 1X antibiotic-antimycotic solution. The rings were incubated at 37°C with 5% CO_2 for 18h. Following incubation, tracheal rings were removed from culture and mounted on to a DMT force transducer for measuring NMDA-induced tracheal smooth muscle response. Untreated tracheal rings cultured for the same time period were used as controls. At the end of each experiment, tracheal rings were washed twice with K-H bicarbonate solution and preserved for RNA analysis.

3.10.0 HDM-INDUCED MURINE MODEL OF ACUTE LUNG INFLAMMATION

3.10.1 – Protocol of HDM-induced lung inflammation in mice

Mice were exposed to HDM exclusively through intranasal (I.N) route. For this purpose, HDM was dissolved in 1X saline solution, with each animal receiving ~35µL containing 0.75µg HDM/µL of allergen equally between each of the nostrils. To facilitate delivery, mice were briefly anesthesized with isoflurane (5%) mixed with O_2. This enabled placement of allergen drops gently on top of the nostrils to facilitate inhalation of HDM, thus ensuring its direct delivery to the lungs. For inducing airway inflammation and AHR, extended acute-allergen exposure protocol was followed. In this, animals were exposed for 5 consecutive days, followed by 48h "rest", and then another 5 consecutive days of challenge. 48h after last I.N administration, lung function parameters were measured. Naive mice, which did not receive any allergen exposure, were used as control.

3.10.2 – Measurement of Lung mechanics: NMDA Concentration-Response Studies

Lung function measurements in naive and HDM-challenged mice were performed on a flexiVENT small animal ventilator (Scireq Inc, Montreal). For this, mice were anesthesized with an intraperitoneal injection of sodium pentobarbital (90mg/kg). Trachea was exposed carefully and mid-cervical tracheostomy was performed by inserting a 20-guage polyethylene catheter into the tracheal opening. The catheter was coupled to the flexiVENT. Mice were ventilated with a tidal volume of 10mL/kg body weight, 150 times per minute with an inspiration: expiration time ratio of 1:1.5. A positive end expiratory pressure (PEEP) of $3cmH_20$, achieved by submerging the expiratory outlet from the ventilator into a water trap, was used for all studies. Once equilibrated on the ventilator, mice were subjected to a serial aerosol NMDA challenge protocol to assess concentration response characteristics of respiratory mechanics. Prior to delivery of each concentration of NMDA, the loading history of the lung was normalized by inflating to total lung capacity and then allowing deflation to functional residual capacity. For NMDA challenge, ~35μL of saline containing 0 to 1mM NMDA was delivered over 10 seconds using an in-line ultrasonic nebulizer. Measurement of respiratory mechanics in response to each dose of NMDA was initiated within 10 seconds of completing the aerosol challenge, with total measurement duration of 5 minutes thereafter. After challenge and the measurement period for each challenge, the next dose was administered until the maximum concentration of NMDA was attained. Following NMDA dosage, a 3mg/mL dose of methacholine was administered as a positive control.

To assess the effects of NMDA challenge on respiratory mechanics, low frequency forced oscillation technique was used. In this, mechanical ventilation was interrupted,

followed by applying a volume perturbation signal using a flexiVENT Prime-8 protocol. The 8-second perturbation signal comprised of 18 serial sine waves spanning a frequency range of 0.25 Hz to 19.625 HZ. In total, 20 perturbations were completed during the course of each 5-minute measurement period. Respiratory mechanical input impedance (*Zrs*) was derived from the displacement of the ventilators piston and the pressure in its cylinder. Correction for gas compressibility and resistive and accelerative losses in ventilator, tubing and catheter were performed according to manufacturer's instructions, using dynamic calibration data obtained from volume perturbations applied to the system in an open and closed configuration. By fitting *Zrs* to the constant phase model, flexiVENT software calculated conducting airway resistance (R_{aw}), peripheral tissue and airway resistance (G) and tissue elastance or stiffness (H). Each parameter was normalized according to body weight. Values for each parameter were calculated as the mean of all 20 perturbation cycles performed after each challenge.

3.11.0 STATISTICAL ANALYIS

Data is presented as means $\pm$ SEM (standard error of mean), unless otherwise stated. For all studies, data was obtained from experiments done at least 3 times or more. Statistical significance was determined by two-way ANOVA with Bonferroni's post-hoc tests, one-way ANOVA with Bonferroni's multiple comparison tests or unpaired student *t*-test, as required by data. *P* values < 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism Software (version 5.0)

CHAPTER 4

NMDA RECEPTORS MEDIATE CONTRACTILE RESPONSES IN HUMAN AIRWAY SMOOTH MUSCLE CELLS

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V.A generated 100% of the experimental data and drafted the manuscript.

4.1.0 ABSTRACT

Human airway smooth muscle (HASM) exhibits enhanced contractility in asthma. Inflammation is associated with airway hypercontractility, but factors that underpin these features are not fully elucidated. Glutamate toxicity associated with increased plasma glutamate concentrations was observed in airway inflammation, suggesting that multi-subunit glutamate receptors, *N*-methyl-*D*-aspartate receptors (NMDA-R) contribute to airway hyperreactivity. We tested the hypothesis that HASM expresses NMDA-R subunits that can form functional receptors to mediate contractile responses to specific extracellular ligands. In cultured HASM cells we measured NMDA-R subunit mRNA and protein abundance by quantitative PCR (qPCR), immunoblotting, flow cytometry and epifluorescence immunocytochemistry. We measured mRNA for a number of NMDA-R subunits, including the obligatory NR1 subunit, which we confirmed to be present as a protein. *In vitro* and *ex vivo* functional NMDA-R activation in HASM cells was measured using intracellular calcium flux (Fura-2AM), collagen gel contraction assays and murine thin cut lung slices (TCLS). NMDA, a pharmacological glutamate analog, induced cytosolic calcium mobilization in cultured HASM cells. We detected 3 different temporal patterns of calcium response, suggesting presence of heterogeneous myocyte subpopulations. NMDA-R activation also induced airway contraction in murine TCLS and soft collagen gels seeded with HASM cells. Responses in cells, lung slices and collagen gels were mediated by NMDA-R as they could be blocked by D-AP5, a specific NMDA-R inhibitor. In summary, we reveal the presence of NMDA-R in HASM that mediate contractile responses via glutamatergic mechanisms. These findings suggest that

accumulation of glutamate-like ligands for NMDA-R associated with airway inflammation contribute directly to airway hyperreactivity.

4.2.0 INTRODUCTION & RATIONALE

Airway smooth muscle (ASM) encircles the airways [1], providing mechanical support and regulating airway caliber [74]. Inflammation-induced alterations in ASM structure and function contribute to variable bronchial airflow obstruction and persistent airway hyperresponsiveness (AHR) in asthma [265-268]. In the airways, multiple mechanisms regulate ASM phenotype and function, including neuroimmune interactions [80, 149], primarily mediated through the release of various neuromediators such as glutamate

Glutamate, a predominant excitatory neurotransmitter in the brain [216] signals via various glutamate receptor classes, broadly categorized as metabotrophic (mGlu-Rs) and ionotrophic (iGlu-Rs). Best studied among the iGluRs are *N*-methyl-*D*-aspartate receptors (NMDA-R), associated with neurodevelopmental and neurodegenerative processes [216, 269]. They exist as transmembrane channels permeable to cations, particularly Ca^{2+} [193, 216, 269]. Functional NMDA-R are multimeric protein complexes comprised of two obligatory NR1 subunits (GRIN1) and two NR2 (GRIN2A-D) subunits or a combination of NR2 and NR3 (GRIN3A-B) subunits [193]. Subunit composition of the receptor determines its specific physiological and pharmacological properties [193, 216, 269].

Classically, glutamate is released from presynaptic nerve endings during excitatory neurotransmission. However, recent evidence suggests immune cells like neutrophils [161], dendritic cells [160], macrophages [270] and platelets [271] release glutamate endogenously. Additionally, functional glutamatergic signaling and NMDA-R expression has been reported in various pulmonary cells and tissues, including alveolar macrophages [244], alveolar Type II cells [253], and the trachea and airways [241, 244, 272]. Nevertheless, information is limited about the functional relevance for NMDA-R in the respiratory system. *In vitro* and *in vivo* observations by Said *et al* [79, 273] suggest an involvement of glutamate and NMDA-R in lung injury and inflammation. In perfused guinea pig tracheal segments NMDA induces edema, augments resting muscle tone and enhances airway responsiveness to cholinergic agonists [79, 244, 273]. Strapkova *et al* [256] have suggested that NMDA-R contribute to airway hyperreactivity as augmentation in ovalbumin-induced tracheal responses to acetylcholine (ACh) are observed in animals that receive NMDA, a pharmacological glutamate analog specific to NMDA-R. Ligand binding to NMDA-R can also promote oxidative stress and lung inflammation in a rat model for experimental sepsis, via a pathway that is blocked by a non-competitive NMDA-R antagonist, MK-801 [274]. Despite these reports, there has been no evidence for a direct role of glutamatergic signaling via NMDA-R in human ASM.

Considering the primary role of ASM in controlling airway responses in asthma [275, 276], we tested the hypothesis that human ASM (HASM) expresses NMDA-R subunits that can form functional receptors to mediate contractile responses to specific extracellular ligands. In this study, using cultured HASM cells we profile NMDA-R

subunit expression using qPCR, immunoblotting, flow cytometry, and immunocytochemistry. We further assessed the capacity of NMDA-R to mediate contractile responses of HASM to the glutamate analogue, NMDA in the presence and absence of the NMDA-R inhibitor D-AP5, by measuring: intracellular Ca^{2+} mobilization in Fura-2-loaded HASM cells; contraction of soft collagen gels seeded with HASM cells; and, airway narrowing using video-microscopy and murine thin cut lung slices.

4.3.0 RESULTS

4.3.1 ASM cells express subunits for complete NMDA-R complexes

To profile NMDA-R subunit expression in cultured HASM cells, we first performed qPCR using subunit-specific primers. We revealed the presence of mRNA for most NMDA-R subunits, including NR1, NR2C, NR2D, NR3A and NR3B, but we found no evidence for NR2A or NR2B. (Fig 4.1A, n=6). The abundance of mRNA for NMDA-R subunits appeared to be stable as we observed no change when cells were serum-deprived for up to 48h (Fig 4.1B, n=6). These data indicate that HASM cells express mRNA for all the subunits required to form a functional NMDA-R complex.

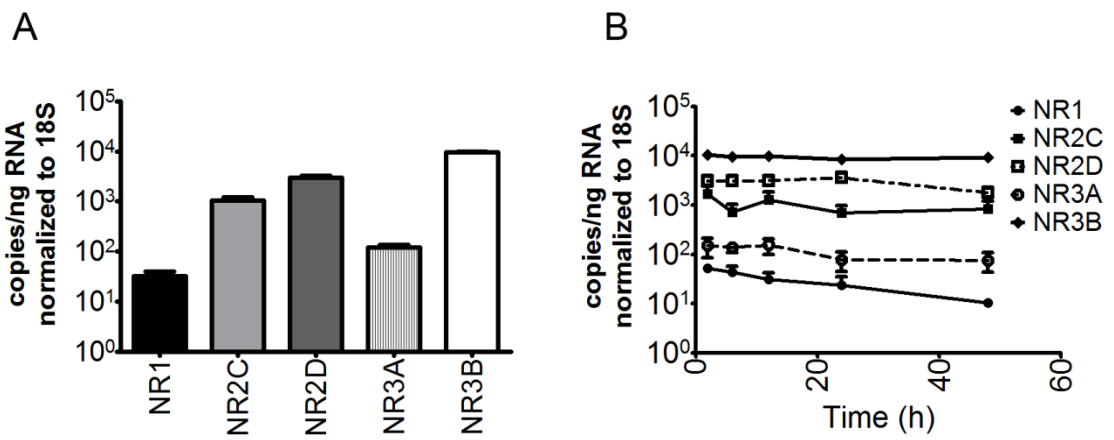


Figure 4.1: *HASM cells express mRNA for different NMDA-R subunits.* qPCR was performed for mRNA isolated from cultured HASM cells. Abundance is expressed as copies/ng RNA normalized to 18S rRNA, (*A)* Abundance of mRNA for respective NMDA-R subunits in confluent HASM cultures maintained in serum-free medium, for 48h. *(B)* Time-course for mRNA abundance of NR1, NR2C, NR2D, NR3A and NR3B in confluent HASM cultures in serum-free conditions. Data represent mean values from 6 different experiments performed in triplicate under similar experimental conditions. Error bars are shown as mean $\pm$ SE.

To validate our qPCR data we next examined the abundance of protein for the obligatory NR1 subunit, essential for formation of functional NMDA-R complexes. Using immunoblotting (Fig 4.2A), NR1 was readily detected (~120kDa) in whole cell lysates from HASM cultures. We noted a modest difference in the mobility of the protein band for NR1 from HASM compared to that for our positive control, mouse brain; this is likely due to tissue- and species-specific differences in glycosylation and other post-transcriptional or translational modifications [277-279]. We further assessed NR1 protein abundance in permeabilized HASM cells using flow cytometry (Fig 4.2B). Fluorescent labeling with an anti-NR1 polyclonal antibody and AlexaFluor488-conjugated secondary antibody revealed significant positive staining, with ~77% of cells exhibiting greater fluorescence compared to cells labeled with isotype-matched control primary antibody. Last, we performed

fluorescence cytochemistry using confocal microscopy, revealing labeling for NR1 in perinuclear regions, the cytoplasm and some foci aligned with cell margins that was absent in fixed and permeabilized HASM cells that were fluorescently immunolabelled with isotype-matched control antibody (Fig 4.2C).

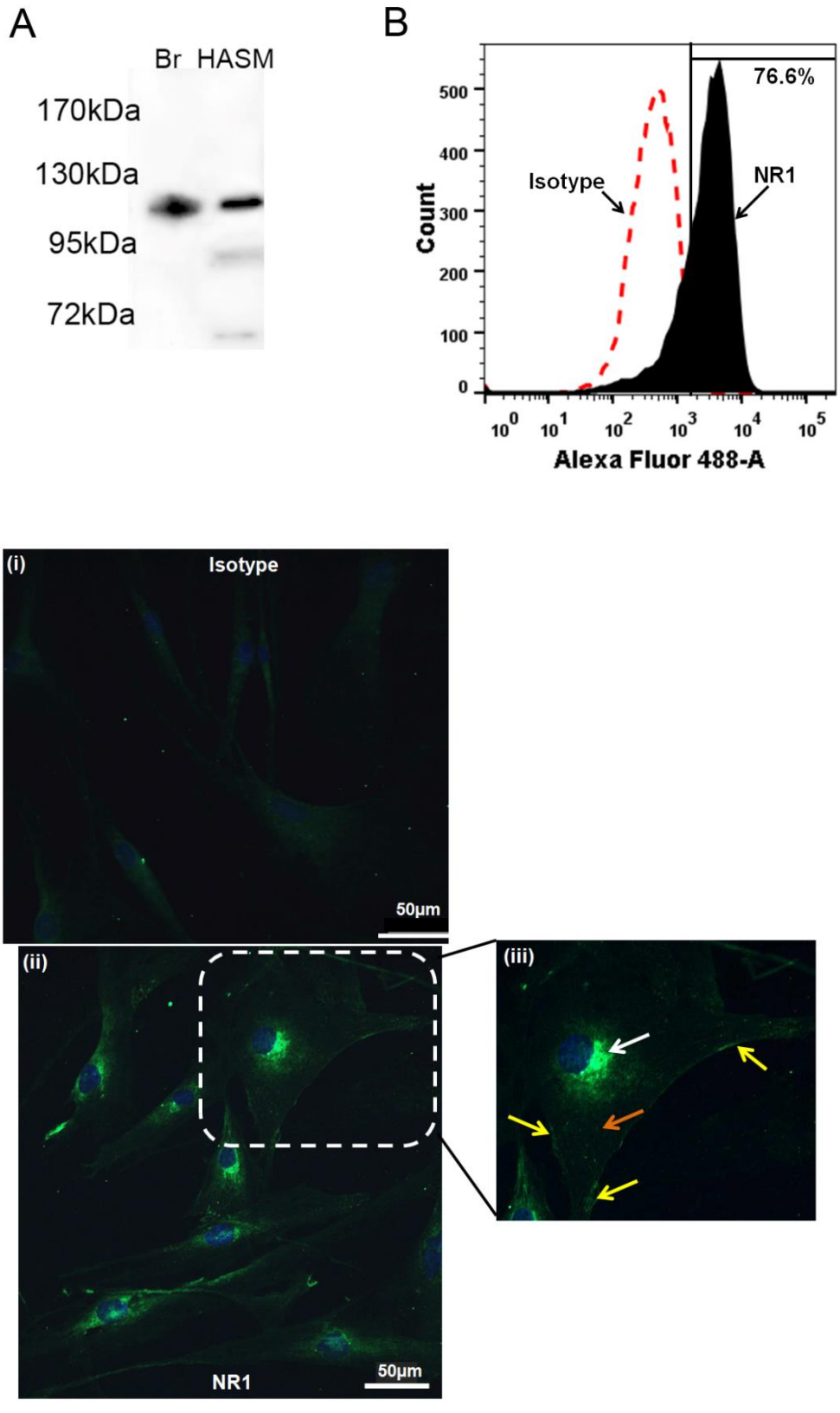
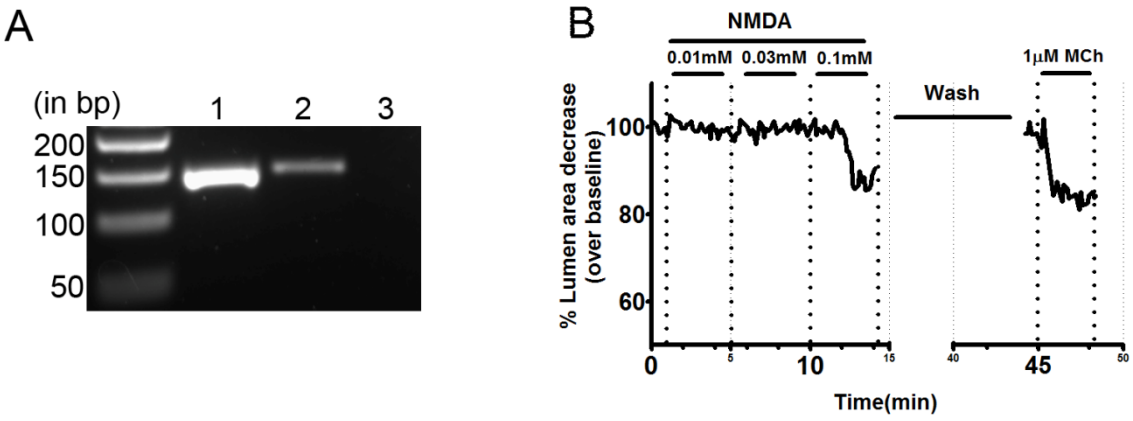

A
Br HASM
170kDa
130kDa
95kDa
72kDa
B
76.6%
Isotype
NR1
Count
Alexa Fluor 488-A
(i) Isotype
50μm
(ii)
NR1
50μm
(iii)

Figure 4.2: *Presence of protein for the obligatory NR1 subunit in HASM cells. (A)* Representative immunoblot from three independent experiments for NR1 (~120kDa band) in protein lysates from HASM cells and from whole mouse brain (Br), which served as a positive control. *(B)* Histogram showing results from flow cytometry of permeabilized HASM cells for NR1 protein (black filled histogram) and IgG isotype control (open histogram, red line). Compared with matched isotype control cells (dotted-lined open histogram), NR1 was detected on ~77% of cells (filled histogram). The histogram shows % mean fluorescence intensity from 10,000 cells and is representative of 5 experiments. *(C)* Confocal micrographs showing immunofluorescence for NR1 in HASM cells (ii). Isotype goat IgG antibody was used as negative control (i). Image in (iii) is an enlarged portion of the boxed region in panel (ii). Arrows point to perinuclear (white), cytosolic (orange) and membrane (yellow) staining. DAPI was used to stain nuclei. Images are representative of 5 independent experiments done under similar conditions. Scale bar: 50μm.

4.3.2 NMDA-R mediates airway contraction, ex vivo

We used murine TCLS preparations, and time-lapse video microscopy to measure intact airway narrowing mediated via NMDA-R. For these experiments we used NMDA, because it mimics glutamate pharmacologically but is selective for the ionotrophic NMDA-R, whereas glutamate is more promiscuous, being a ligand for both mGluRs and iGluRs. Prior to completing these functional studies, we surveyed NR1 subunit expression in mouse lungs using RT-PCR and detected a ~137bp transcript corresponding to NR1 (Table 1 and Figure 4.3A). Using a cumulative NMDA dose-response experimental design in the presence of 0.2mM glycine, we measured airway narrowing in TCLS (Fig 4.3B and 4.3C). We did not observe any significant reduction in airway lumen area from baseline in response to 0.01 or 0.03mM NMDA, but at the highest concentrations tested (0.1 and 0.5mM) NMDA significantly narrowed airways by 10%. Maximum constriction was achieved with 0.1mM NMDA as no increase was evident using 0.5mM NMDA (not shown), thus for subsequent functional studies we used 0.1mM NMDA. To confirm that NMDA-induced airway narrowing was mediated by NMDA-R, some TCLS were pre-

incubated with the selective NMDA-R antagonist, (2R)-amino-5-phosphonopentanoate (D-AP5; 0.5mM). We found that D-AP5 abrogated NMDA-induced airway narrowing (Fig 4.3C). In all TCLS specimens we also determined active narrowing in response to 1μM methacholine (MCh) as a comparator to that induced by NMDA. We found that NMDA-induced contraction (0.1mM) was physiologically significant, being ~50% of that induced by MCh (Fig. 4.3C). To further characterize the impact of NMDA on airway contraction, we compared airway narrowing in response to 1μM MCh in TCLS before and after NMDA exposure (Fig 4.3D). We did not observe any effect of NMDA exposure on MCh-induced airway contraction.



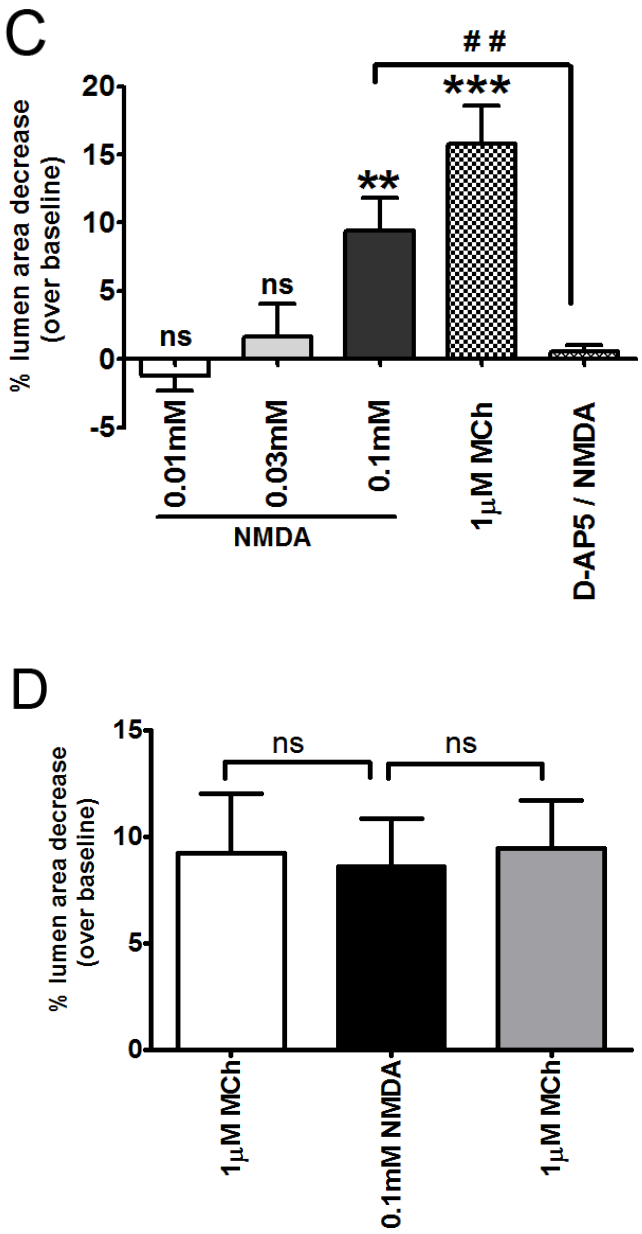
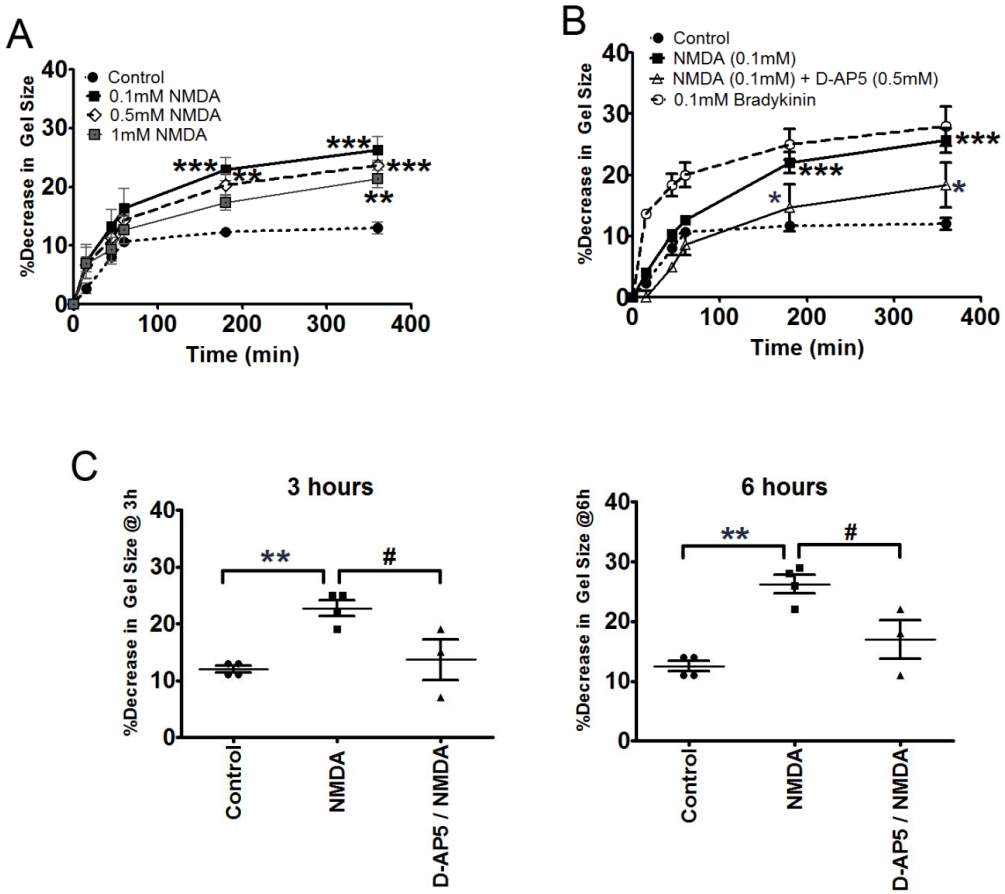


Figure 4.3: *Agonist-induced NMDA-R activation causes airway contraction. (A)* Agarose gel showing products generated by RT-PCR using primers for the obligatory NR1 subunit in mouse brain (lane 1) and mouse lungs (lane 2). Lane 3 is a negative control (water). The left lane shows size markers as indicated. Equal volume of sample (2.5µl) was loaded in a 2% agarose gel. *(B)* Representative tracing from a TCLS showing temporal pattern of active narrowing of lumen area of a single airway perfused with increasing concentrations of NMDA. At the end of each study TCLS were perfused with saline for (30 min (wash) then re-stimulated with 1µM MCh. Response to each agonist was recorded for 4 min. Experimental values were normalized as % decrease in baseline (pre-agonist) lumen area (Y–axis). *(C)* Histogram showing cumulative dose-response

patterns for airway narrowing in response to increasing concentrations of NMDA or to 1μM MCh. In some studies TCLS were pre-incubated with 0.5mM D-AP5 prior to stimulation with 0.1mM NMDA (D-AP5/NMDA). Data presented represent the mean ± SE from 13-16 airways obtained from 5 different mice. Data was analyzed by One-way ANOVA. **P<0.01, ***P<0.001 and ns (not significant) compared to baseline). ## P<0.01 for D-AP5/NMDA compared to 0.1mM NMDA. (D) Histogram summarizing the mean ± SE for airway narrowing in response to 1μM MCh before (white) and after (grey) treatment with 0.1mM NMDA. Response to NMDA alone is also shown (black). Airways were perfused with saline for at least 30 minutes between agonist additions to ensure that airway lumen area returned to baseline (unstimulated). Data is the mean from 9 separate airways from 4 different mice, analyzed by One-way ANOVA (ns=not significant).

To assess the direct effects of NMDA on HASM contraction, we next used floating collagen gels seeded with HASM cells and measured the decrease in collagen gel area that occurs upon NMDA exposure (Figure 4.4). Compared to control gels that were not exposed to agonist, NMDA (0.1, 0.5 and 1mM) induced significant contraction of HASM-seeded collagen gels, reaching near-maximum shortening (~20% decrease in gel size) after 60min, a response that was stable for up to 6h (Fig 4.4A and 4.4C). Interestingly, all concentrations of NMDA triggered gel contraction of similar magnitude suggesting that using this system NMDA-R mediates HASM cell contraction in an all-or-none manner. To confirm that NMDA-R mediates HASM contraction, we pre-treated some gels with the NMDA-R antagonist, D-AP5, and found that contractile responses were blocked (Figs. 4.4B and 4.4C). We also compared collagen gel contraction induced by NMDA (0.1mM) with that of a physiologically relevant positive control, bradykinin (0.1mM) and found that though NMDA-induced contraction was of a slower onset, its magnitude was not different from that induced by bradykinin (Fig 4.4B). We also tested contractile response to MCh (0.1mM) and to histamine (0.1mM), considered to be physiologically relevant G_q-coupled agonists known to induce robust contraction in intact airways (Fig 4.4D). MCh-elicited gel contraction was comparable to that induced by bradykinin, and histamine also induced

significant contraction, albeit to a lesser degree than other agonists. Further, we showed that contraction of collagen gels over time requires the presence of airway myocytes (Fig 4.4D). In comparison to gels that harbored HASM cells, cell-free collagen gels demonstrated no more than ~3% reduction in their area over a 5h time period, with gel contraction being almost "0" in the first 3hours. Thus, collagen gels themselves do not undergo any significant physical change, thus confirming that the contractile effect observed in our experiments is mediated by HASM cells.



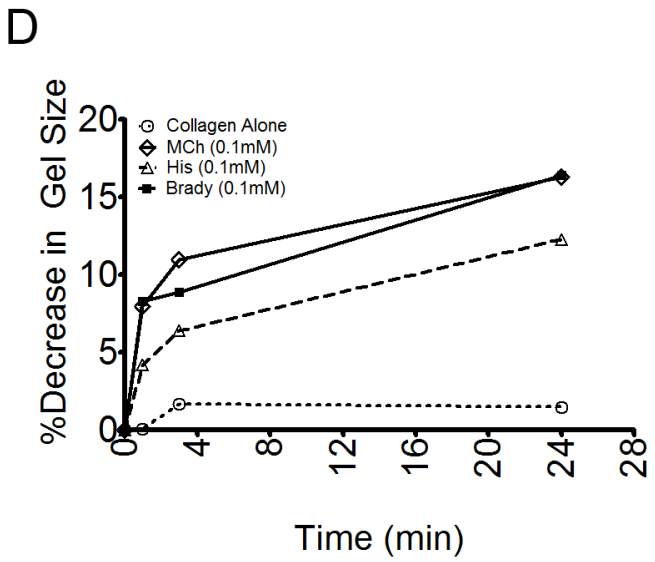
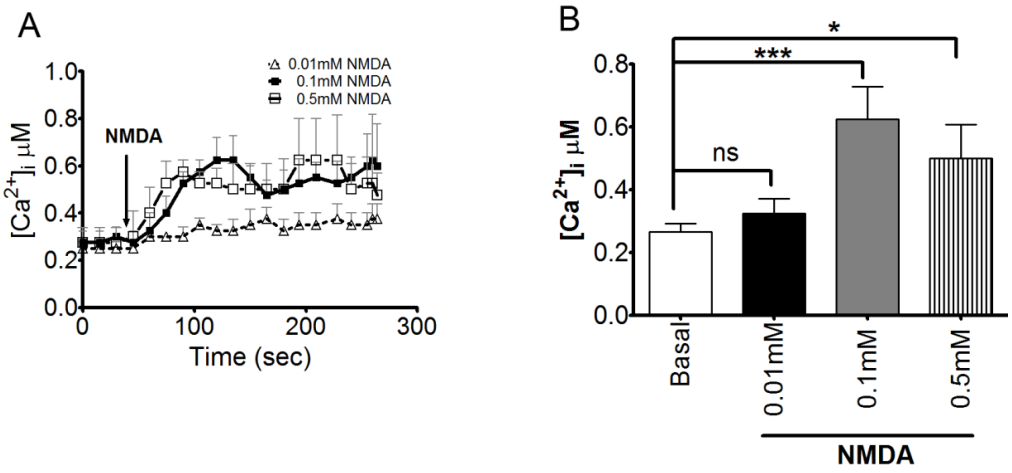


Figure 4.4: *NMDA-R directly mediates contraction of HASM cells.* (A) Contraction of collagen gels seeded with HASM cells stimulated with increasing concentration of NMDA or retained in culture medium alone (Control). Data for each time point is the mean + SE of 3 independent experiments done in triplicate. Comparisons were made using two-way ANOVA with Bonferroni's post hoc test. *** P<0.001; and, ** P<0.01 compared to Control at each time point. No statistical difference existed between time-matched values for gels exposed to 0.1mM, 0.5mM or 1mM NMDA. (B) Time response curve showing effect on collagen gel contraction before and after treatment with NMDA-R antagonist D-AP5. Gels were either untreated (Control) (solid dots), pre-treated with 0.5mM D-AP5 for 2h then stimulated with 0.1mM NMDA (open triangles), or treated with 0.1mM NMDA alone (solid squares) then gel area size was measured. As a positive control some gels were treated with 0.1mM bradykinin (open circles). Data for each time point is the mean + SE of 3 experiments done in triplicate. Comparisons were made using two-way ANOVA with Bonferroni's post hoc test. ***, P<0.001; and *, P<0.05) compared to Control at each time point. (C) Scatter plots showing percentage decrease in collagen gel area at 3h (left) and 6h (right) in Controls (solid circles), gels treated with 0.1mM NMDA (solid squares) or 0.5mM D-AP5/0.1mM NMDA (solid triangles). Data were obtained from 3 independent experiments. Mean values for each group is designated as a horizontal line. Data were analyzed by One-way ANOVA with Bonferroni's multiple comparison test. **P<0.01 for 0.1mM NMDA alone compared to Control and $^{\#}$ P<0.05 for D-AP5/NMDA compared to 0.1mM NMDA. (D) Time response curve showing contraction in cell free collagen gels (open circles, dotted line), and gels containing HASM cells stimulated with 0.1mM MCh (open diamonds), 0.1mM histamine (open triangles, dashed line) or 0.1mM bradykinin (filled squares). Values are representative of 2 experiments.

4.3.3 NMDA-R mediates a heterogeneous pattern of $[Ca^{2+}]_i$ flux in HASM cells

Upon agonist binding, NMDA-R activation causes influx of extracellular Ca^{2+} in neurons [216], therefore, we used real-time Ca^{2+} imaging to examine whether NMDA-R mediates Ca^{2+} flux in HASM cells. HASM exhibited a significant increase in $[Ca^{2+}]_i$ in response to both 0.1mM and 0.5mM NMDA, but we observed no change in $[Ca^{2+}]_i$ in response to 0.01mM NMDA (Figure 4.5A and 4.5B). NMDA induced a sustained calcium response over a period of 4 min attaining peak $[Ca^{2+}]_i$ of ~0.6µM after 120s (Fig 4.5A and 4.5B). The elevation in $[Ca^{2+}]_i$ induced by NMDA was abrogated when cells were pre-treated with D-AP5 (0.5mM), confirming that NMDA-R mediates changes in $[Ca^{2+}]_i$ (Fig 4.5C and 4.5D). We compared response to NMDA with that triggered by 0.1mM bradykinin and found that: time-to-peak $[Ca^{2+}]_i$ was much slower; peak $[Ca^{2+}]_i$ was ~65% lower than the acute peak induced by bradykinin; and peak $[Ca^{2+}]_i$ induced by NMDA was sustained and similar in magnitude to that sustained in response to bradykinin (Fig 4.5C). Notably, NMDA-induced Ca^{2+} flux in HASM cells was observed only in the presence of glycine (0.2mM), a NMDA-R co-agonist. Without glycine, NMDA failed to evoke any calcium response in our cell preparations (Fig 4.5D), which confirms the necessity of simultaneous binding of agonist (NMDA) and co-agonist (glycine) to evoke NMDA-R activation. To determine whether calcium mobilization mediated by NMDA-R was reliant on extracellular calcium, we measured changes in $[Ca^{2+}]_i$ induced by NMDA in cultured HASM cells incubated in Ca^{2+}-free buffer. In the absence of extracellular Ca^{2+}, we observed no increase in $[Ca^{2+}]_i$ upon NMDA exposure (Fig 4.5E). These data indicate that NMDA-R can mediate increase in cytosolic Ca^{2+}, and that extracellular Ca^{2+} is required for this effect. To further complement our findings, we examined $[Ca^{2+}]_i$ elevation in HASM cells when stimulated with endogenous NMDA-R ligand, glutamate in the presence

and absence of co-agonist glycine. We used a physiologically relevant concentration of glutamate (0.1mM) and observed that it evoked significant Ca^{2+} mobilization (~1.2µM above baseline) in the presence of glycine. Notably, glutamate induced Ca^{2+} responses were observed even in the absence of glycine; however, the active release of Ca^{2+} was ~40% less than in the presence of glycine. This suggests that NMDA receptor accounts for 60% of the glycine-dependent glutamate response. Consistent with this, we observed that Ca^{2+} mobilization by glutamate in the presence of glycine was also blunted by ~40% when cells were treated with the 0.5mM D-AP5 (Fig 4.5F). Together, these observations validate our findings with NMDA, but also suggest the presence of non-NMDA glutamate receptors in HASM cells.



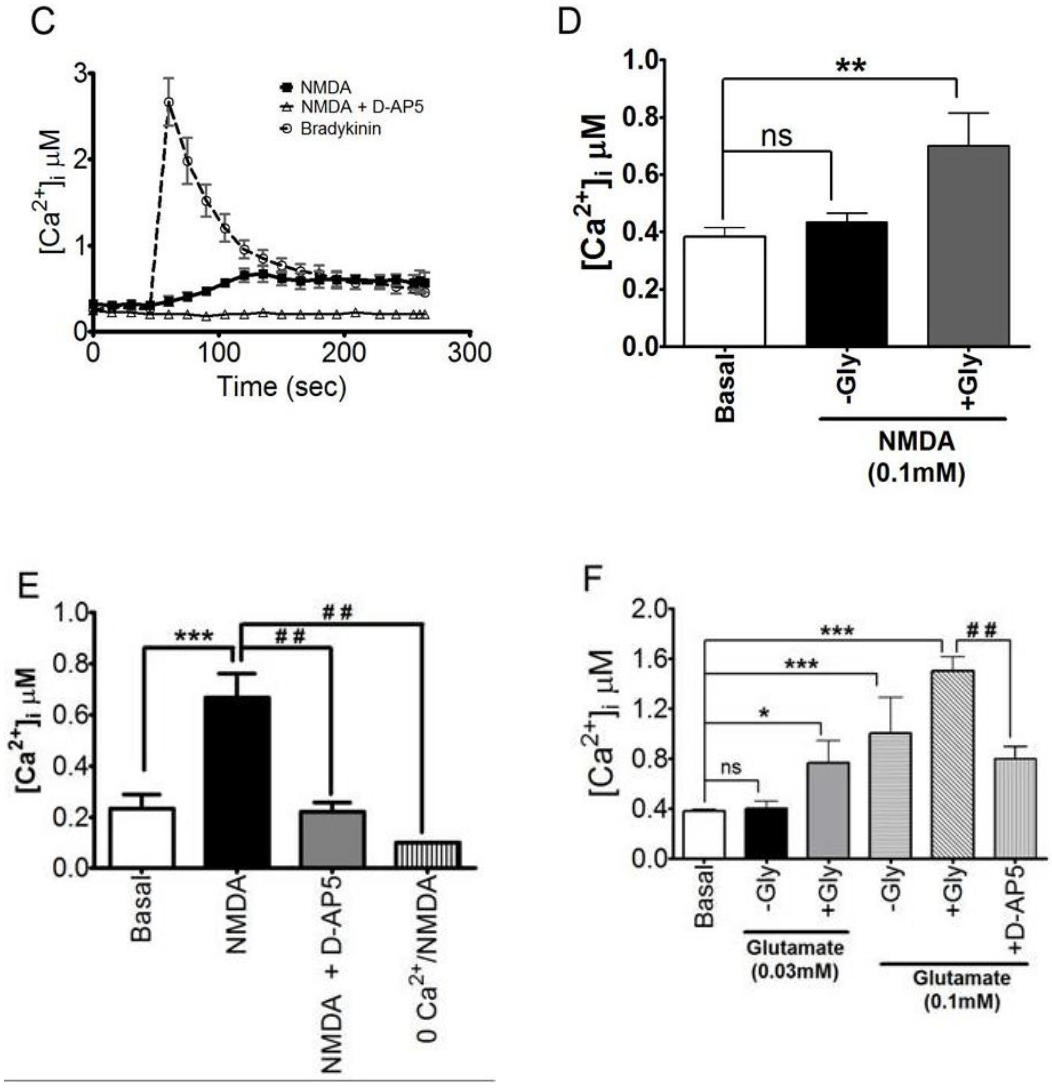
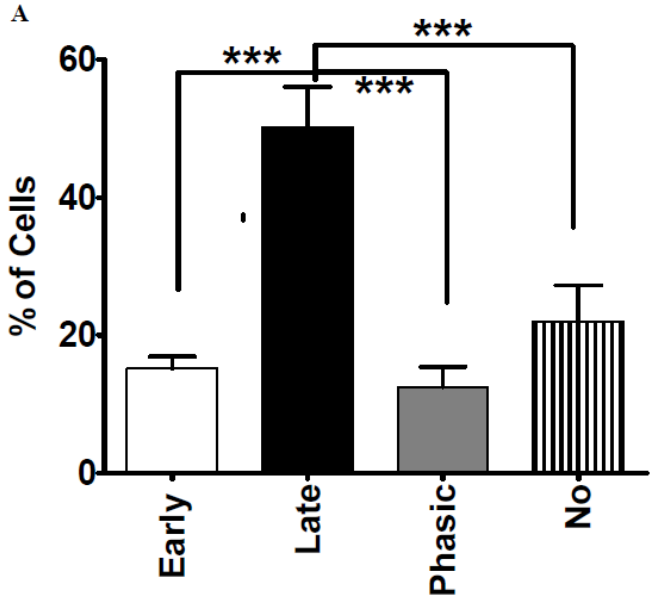


Figure 4.5: *NMDA-R induces Ca^{2+} flux in HASM cells*. *(A)* Cultured HASM cells were serum-deprived for 48h, loaded with Fura-2AM, then temporal changes in free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured by fluorescence microscopy. Cells were stimulated with increasing concentrations of NMDA: 0.01mM (open triangles), 0.1mM (solid squares) and 0.5mM (open squares). $[Ca^{2+}]_i$ was recorded for 4 minutes. Basal $[Ca^{2+}]_i$ was determined prior to addition of agonists The graph shows mean data + SE from 50-60 cells present within the microscopic field from HASM cultures in 4 separate experiments. *(B)* Histogram showing mean values +SE for basal (white) and maximum $[Ca^{2+}]_i$ observed at 135s when HASM cells were stimulated with 0.01mM (black), 0.1mM (grey) and 0.5mM NMDA (lined). Values were determined from 4 independent experiments done in duplicate and analyzed by one-way ANOVA with Bonferroni's multiple comparison test: ***P<0.001; *P<0.05; and ns (not significant) compared to basal. *(C)* Mean temporal tracings showing $[Ca^{2+}]_i$ in HASM cells exposed to NMDA (0.1mM), pretreated with 0.5mM D-AP5 (2h) before exposure to NMDA (0.1mM); or treated with bradykinin (0.1mM). Data is from 5 independent experiments done in duplicate. (*D*) Histogram showing mean values +SE for basal (white) and maximum $[Ca^{2+}]_i$ observed when HASM cells were stimulated with

0.1mM NMDA in the absence (black) and presence (grey) of 0.2mM glycine. Values were determined from 3 independent experiments and analyzed by one-way ANOVA with Bonferroni's multiple comparison test: **P<0.01; and ns (not significant) compared to basal. *(E)* Histogram summarizing mean data +SE for $[Ca^{2+}]_i$ under basal conditions (white), or as maximum $[Ca^{2+}]_i$ in HASM cells treated with NMDA (0.1mM) (black), pre-treated with D-AP5 (0.5mM) prior to NMDA (0.1mM) exposure (grey), or cultures pre-incubated in Ca^{2+}-deficient buffer prior to treatment with NMDA (0.1mM) (lined). Data were obtained from 5 independent experiments done in duplicate and analyzed by One-way ANOVA with Bonferroni's multiple comparison test. ***, P<0.001 compared to Basal, and ##, P<0.01 compared to NMDA (0.1mM). (*F*) Histogram summarizing mean data + SE for $[Ca^{2+}]_i$ under basal (white), or as maximum $[Ca^{2+}]_i$ in HASM cells treated with 0.1mM glutamate without glycine (black), or with 0.2mM glycine in the absence (grey) and presence of 0.5mM D-AP5 prior to glutamate (0.1mM) exposure (vertical lined). Data were obtained from 3 independent experiments done in duplicate and analyzed by One-way ANOVA with Bonferroni's multiple comparison test. ***, P<0.001; compared to basal, and #, P<0.01 compared to glutamate (0.1mM, + glycine).

In all cultures studied, NMDA alone routinely induced an elevation in $[Ca^{2+}]_i$ in 75-80% of cells, and there were no significant differences in basal $[Ca^{2+}]_i$ between cells. However, in the course of our experiments using real time fluorescent microscopy to measure changes in $[Ca^{2+}]_i$, we observed that individual cells exhibited a heterogeneous temporal response pattern that we have classified broadly into four groups: Early Response; Late Response; Phasic Response; and, No Response (Fig 4.6). Consistent with the mean Ca^{2+} response curve for the whole culture population (see Fig 4.5A), ~50% of all cells were of the Late Response group (Fig 4.6A), displaying a delayed (~60-100s after NMDA exposure) Ca^{2+} response that was sustained for at least 300s (Fig 4.6B (iii and iv)). A sub-group we identified as showing a Phasic Response profile (initial acute rise in $[Ca^{2+}]_i$ within 20s followed by a second spike 75-100s later) (Fig 4.6B (vi)), encompassed approximately 13% of all myocytes (Fig 6A). Indeed, some cells even presented 3-5 calcium spikes within the timeframe of measurement (300s) (Fig 4.6B (v)). We also identified 15% of cultured HASM as exhibiting an Early Response profile characterized by an almost immediate

response (10-15s after NMDA exposure) (Fig 4.6A and 4.6B (i and ii)). Finally, we observed that 22% of the total cell population did not show any change basal $[Ca^{2+}]_i$ upon NMDA, thus forming the so-called No Response sub-group (Fig 4.6A). NMDA-induced increases in $[Ca^{2+}]_i$ in all response sub-groups were blunted by removal of extracellular Ca^{2+} or selective antagonism of NMDA-R with D-AP5 (data not shown). Together, these results demonstrate an inherent heterogeneity in NMDA-R mediated mobilization of cytosolic Ca^{2+} in sub-groups of HASMs that is dependent upon extracellular Ca^{2+}, suggesting there could be differential expression of NMDA-R subunits, regulation of NMDA-R function, or both.



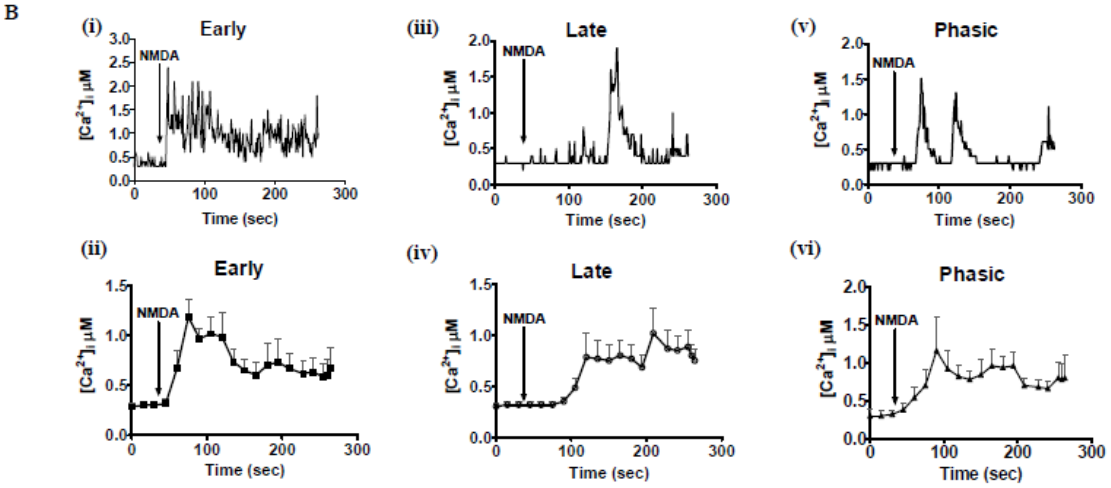


Figure 4.6: *HASM cells exhibit heterogeneous temporal patterns for mobilization of intracellular Ca^{2+} upon NMDA exposure*. (A) Histogram showing distribution (%) of cells exhibiting different temporal responses for Ca^{2+} mobilization in response to 0.1mM NMDA (examples shown in 6B). Tracings from ~30 randomly selected cells in a field were analyzed from 5 independent experiments done in duplicate and individual cell response was categorized into one of four groups: *Early Responses* (white), *Late Responses* (black), *Phasic Responses* (grey) or *No Response* (lined). Analysis was done using One-way ANOVA with Bonferroni's multiple comparison test *(B)* Tracings showing representative (i, iii and v) and mean (ii, iv and vi) Ca^{2+} responses upon NMDA stimulation for different cell populations. Following agonist stimulation, cells showing Early responses (i and ii) mobilized $[Ca^{2+}]_i$ within 10s, Late responses (iii and iv) showed a response 100s later and Phasic responses (v and vi) showed Ca^{2+} spikes respectively. Each mean curve represents data collated from at least 20 individual cells from 5 independent experiments. Error bars show + SE.

4.4.0 DISCUSSION

Ours is the first study to provide direct evidence for NMDA-R expression by HASM cells and that these receptors can mediate contraction by inducing an elevation of intracellular Ca^{2+}. Using qPCR we show that mRNA for all but two of the known NMDA-R subunits is present in cultured HASMs and we confirm that protein for the obligatory NR1 protein is readily detectable. Our functional analyses show that ligand binding for

NMDA-R leads to increased $[Ca^{2+}]_i$, directly induces contraction of HASM cells, and triggers physiologically relevant degrees of airway narrowing *ex vivo*. Collectively, our data reveals for the first time that requisite subunits can be assembled into functional multimeric NMDA receptor complexes.

As proposed by Haong et al [280], AHR occurs as a consequence of several exogenous and endogenous stimuli, including neurogenic mediators such as glutamate and their receptors like NMDA-R. While glutamate is proposed to be a novel regulator of adaptive T-cell immune responses [251], deregulated ion transport mediated through hyperexcitation of ligand-gated glutamate channels such as NMDA-R either in excitatory or non-excitatory pulmonary cells or tissues is suggested to be an potential mechanism underlying increased airway responsiveness during inflammation [256, 280]. Besides glutamate and its pharmacological analog NMDA, NMDA-R can be activated by other physiological ligands such as D-serine, L-alanine, L-cysteine and quinolinic acid [188]. The presence of multiple allosteric modulators and distinct subunit composition of NMDA-R appears to determine the functional diversity of the receptor [185, 188]. NR2A-D and NR3A-B subunits act as modulatory proteins in the receptor complex [188, 193, 216, 269]. While NR2 harbors the glutamate-binding domain, NR1 and NR3 subunits contain the glycine-binding site; glycine functions as a NMDA-R co-agonist. Studies by several groups, including Dickman *et al* [244], show NR1, NR2C and NR2D subunit expression in rat peripheral lungs. Consistent with these findings, we observed the expression of these NMDA-R subunits in ASM cells, and we also identified the presence of mRNA for NR3 subunits (NR3A and NR3B). Our studies using cultured HASM cells from adult donors

indicated that NMDA-R subunits may be stably expressed by airway myocytes. We found that mRNA abundance was highest for the NR2C, NR2D and NR3B. Differential expression of subunits is consistent with studies in other cell types. For instance, NMDA-R subunit composition and distribution in neurons is plastic and dependent on the CNS developmental stage, neuronal activity and tissue microenvironment [186, 188]. Nonetheless, the presence of protein for the NR1 subunit is indispensable for the formation of functional NMDA-R, and our data confirm that both mRNA and protein for NR1 is abundant in lung tissue and HASM cells. Thus, our findings, aligned with that from other groups on multiple cell and tissue types, indicate a need for additional research to determine the role of various receptor subunits in modulating ASM contractile function.

ASM is essential in regulating airway conductance. Its emergence as a phenotypically diverse tissue that is also capable of orchestrating local inflammation and promoting airway remodeling warrants attention as a potential therapeutic target [11]. Notably, the multifunctional ability of ASM is controlled, in part, through oscillation patterns for $[Ca^{2+}]_i$, which is in turn regulated by various Ca^{2+} pumps and membrane ion channels. We examined whether NMDA-R on ASM cells demonstrate a role in mediating changes in cytosolic Ca^{2+}, an effect that requires extracellular calcium when myocytes are exposed to NMDA. Our studies suggest that NMDA-R may serve as a sarcolemma transmembrane calcium channel in ASM. However, the approach we used, fluorescence microscopy with Fura-2 loaded cells, does not allow direct discrimination of the contribution of Ca^{2+} derived from extracellular or intracellular stores, or whether influx of extracellular Ca^{2+} may trigger release of intracellular stores, thereby leading to the

increases in $[Ca^{2+}]_i$ that we observed upon ligand binding to NMDA-R. Ca^{2+} released from internal sarcoplasmic reticulum induces ASM contraction, while membrane ion channels are typically thought to replenish calcium stores [125]. Our data supports the notion that NMDA-R-mediated changes in cytosolic Ca^{2+} in human ASM cells could contribute directly to airway contraction.

Our experiments revealed that NMDA, only in the presence of glycine, was sufficient to induce maximum increase in $[Ca^{2+}]_i$, which we confirmed using glutamate, a more physiological NMDA-R agonist. NMDA is an amino acid derivative used as a pharmacological analogue of glutamate to specifically distinguish NMDA-R from other glutamate receptors. Considering normal physiological plasma of glutamate concentrations range between 0.05mM and 0.08mM [158, 281], our observations that maximum responses could be induced with 0.1mM NMDA are of physiological relevance. HASM cells, treated with 0.1mM glutamate, exhibited a significant Ca^{2+} mobilization, which was comparable to those induced by 0.1mM NMDA, thereby supporting our results with NMDA and involvement of glutamatergic signaling in airways linked to NMDA receptors. Agonist-binding affinity of NMDA-Rs to NMDA (EC50 35-50µmol L^1) is significantly lower than glutamate (EC50 1-3µmol L^{-1}) that is further dependent on the receptor subunit configuration [282, 283]. During inflammatory conditions such as lung injury, endothelial permeability facilitates extravasation of glutamate towards the inflamed tissues causing an increased local glutamate concentration [158]. In this context, the increases in $[Ca^{2+}]_i$ we observed with 0.1mM NMDA and even higher concentrations (0.5mM) are physiologically relevant as they fall within the levels of plasma glutamate levels that have been measured

for normal and diseased conditions. More importantly, we found presence of glycine as an essential pre-requisite for NMDA-R mediated Ca^{2+} flux in HASM cells. Cells responded to glutamate even without glycine. Our experiments show that unlike responses to NMDA, glutamate-induced $[Ca^{2+}]_i$ responses cannot be blocked completely by D-AP5 suggests likely presence of additional glutamate receptors including mGluRs. In ASM cells, NMDA-induced Ca^{2+} responses are only a quarter of the magnitude of that induced by a robust GPCR agonist such as bradykinin, but NMDA-R mediates sustained elevation in $[Ca^{2+}]_i$ suggesting the potential to activate multiple signaling pathways associated with airway contraction. Our experiments do show that inhibiting NMDA-R with D-AP5 prevents all responses triggered by NMDA-R ligands. This includes abolition of all of the diverse temporal patterns for Ca^{2+} flux that we characterized in HASM cells. Sukkar *et al* [86] suggest that ASM cells of diverse phenotype and with disparate synthetic, contractile and proliferative function are actually representative of a continuum of overlapping cell populations. The heterogeneous intercellular Ca^{2+} responses mediated by NMDA-R that we observed may reflect a composite of multi-functional behavior that is a fundamental character of ASM cells. Also, existence of spatio-temporal heterogeneity in Ca^{2+} oscillations between airway myocyte subpopulations to agonists such as MCh and bradykinin described by Sieck *et al* [284] and by us [257] suggests such a phenomenon is not unique to NMDA-R. Thus, future studies that directly correlate ASM functional capacity with Ca^{2+} flux patterns mediated by NMDA-R, albeit technically challenging, could reveal the full spectrum of control that NMDA-R may contribute to ASM cells.

In humans, increased frequency of $[Ca^{2+}]_i$ oscillatory waves is believed to be the fundamental event underlying agonist-induced contractions initiated by ASM tissue [125]. NMDA-R involvement in promoting airway muscle contractility was previously shown, albeit in animal models [256]. These studies used isolated tracheal rings, which do not necessarily represent ASM responses surrounding smaller airways. To further understand the physiological significance of NMDA-R-mediated $[Ca^{2+}]_i$ influx in ASM cells, we used murine thin cut lung slices to measure airway contraction, a methodology widely accepted to study the behavior of smaller airways [128]. Lung slices retain an *in vivo*-like cellular and tissue microstructure surrounding the airways, which provides an ideal platform to correlate microscopic sub-molecular events with macroscopic responses in a physiologically relevant manner [128]. Using cultured airway slices, we show that NMDA-induces airway contraction via pathways that require NMDA-R, as the NMDA-R antagonist D-AP5 blunted airway constriction. In guinea pig tracheal segments, Said *et al* [79] and others [256] showed that NMDA enhances airway contractile responses to MCh. In our experiments, we found no such positive effect of NMDA when we measured murine airway responses to 1μM MCh before and after NMDA treatment. It is not clear why such a discrepancy between those data and ours might exist, but this could be ascribed to innate disparity between larger and smaller airways or to species-to-species variation. Additionally, we observed considerable variation in airway contractile responses to both NMDA and MCh individually, which corresponds to the biological heterogeneity observed across different *in vitro, ex vivo* and *in vivo* models used in studying airway behavior.

To further ascertain the involvement of human ASM cells in regulation of airway contraction, we employed a collagen gel assay system as a way to measure contraction at cellular level. We show that 0.1mM NMDA decreased collagen gel area by ~20%, a maximum response that reflects an all-or-none effect as lower concentrations elicited no contraction, whereas higher concentrations did not increase the contractile response. Nonetheless, maximum contraction induced by NMDA-R activation was significant as it mimicked that induced by bradykinin, whose ability to contract HASM-seeded gels was comparable to other Gq-coupled contractile agonists MCh and histamine. Furthermore, contractile responses demonstrated were indeed by HASM cells, as cell-free collagen gels failed to evoke any contraction over time. These observations concur with that of Bourke *et al* [285], where authors confirmed that human ASM-mediated collagen gel contraction measured over a time period of 72h, as they saw no physical change in collagen gel lattices that were not seeded with HASM cells. The collagen gel contraction model does have limitations in interpretation to physiological systems, as smooth muscle contraction is generally not rapid, and removal of agonist does not result in relaxation. The gel system does resemble that of Margulis *et al* and others [285, 286] where diminutive, yet noticeable contractile responses generated during wound healing are portrayed over a period of hours to days. In our experiments, bradykinin demonstrated a peak contractile response only after 30min even though $[Ca^{2+}]_i$ flux responses were rapid and occurred within 30s of adding NMDA. Peak NMDA-induced contraction in ASM cells occurred 3h after agonist exposure. Our observations in response to bradykinin are comparable to the kinetics demonstrated in the study by Sutcliffe *et al* [287]. Notably, NMDA-induced reduction in

collagen gel area was inhibited by D-AP5 indicating that the response measured is mediated via NMDA-R.

Our observations using multiple systems are in accordance with those in different small animal models of asthma reported by Said *et al* [273] and Strapkova *et al* [256] in that they support a role for NMDA-R as a contributor to airway hyperreactivity. Together, our studies confirm NMDA-R and glutamatergic signaling to be a potential mechanism for regulating airway contractility, likely through direct effects on $[Ca^{2+}]_i$ in ASM cells. NMDA-R in ASM cells might have additional functional roles that were not addressed in our work, for example whether in conditions of inflammation NMDA-R subunit expression is altered or pathways that regulate NMDA-R function are altered. In summary, we provide evidence that cultured human ASM cells express functional NMDA-R containing the obligatory NR1 subunit. Activation of NMDA-R on ASM cells results in significant increase in $[Ca^{2+}]_i$ that mediates airway myocyte contraction. This presents NMDA-R as a novel regulator of ASM cell function. Nonetheless, the physiological relevance of NMDA-R in healthy and diseased airways needs further investigation.

4.5.0 ACKNOWLEDGEMENTS

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CHAPTER 5

TUMOR NECROSIS FACTOR (TNF) REGULATES NMDA RECEPTOR-MEDIATED HUMAN AIRWAY SMOOTH MUSCLE FUNCTION AND MURINE AIRWAY RESPONSES

(Manuscript under submission)

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V.A performed 90% of the experiments required for the realization of this article, analyzed 100% data and drafted the manuscript.

5.1.0 ABSTRACT

Tumor necrosis factor (TNF) augments smooth muscle mediated airway contractility via influencing regulatory mechanisms involved in the maintenance of cellular calcium homeostasis. We recently showed that *N*-methyl-*D*-aspartate receptors (NMDA-Rs) form a novel receptor-operated calcium entry (ROCE) channel in human airway smooth muscle (HASM) cells that can mobilize intracellular Ca^{2+} and induce airway contraction. Available literature suggests TNF profoundly regulates NMDA-R subunit expression and receptor activity, thereby potentiating glutamate-induced cell death primarily in cortical neurons and glial cells. Considering the importance of TNF in the pathogenesis of chronic airway disorders such as asthma, we tested the hypothesis that NMDA-R mediated Ca^{2+} and contractile responses to extracellular ligands in HASM cells are altered upon exposure to inflammatory cytokines, in particular TNF. Using *in vitro* cultured human airway myocytes exposed to 10ng/mL TNF for 48h, we determined NMDA-R subunit relative abundance by quantitative PCR (qPCR), confocal immunocytochemistry and immunoblotting. We observed a significant dose-dependent and time-dependent increase in obligatory NR1 subunit expression in HASM cells with TNF. Also, TNF modified the mRNA expression of regulatory NR2 and inhibitory NR3 subunits suggesting a changed receptor composition by TNF. Further, we measured intracellular Ca^{2+} flux responses (using Fura-2 AM) in TNF-treated HASM cells to NMDA, a pharmacological glutamate analog specific to NMDA-R. Compared to unstimulated cell population, we observed an enhanced calcium mobilization along with rearrangement of temporal patterns in calcium responses due to NMDA-R activation upon TNF exposure. *Ex vivo* airway responsiveness to NMDA were determined in murine thin cut lung slices (TCLS) exposed to TNF or obtained from a

house-dust mite (HDM) exposed mouse model of allergic asthma. While NMDA-R activation caused considerable airway contraction in unstimulated control slices, we observed an opposite and noticeable dilatory response in both TNF-treated and HDM-exposed airways, which was attenuated with D-AP5, a specific NMDA-R antagonist. Further, NMDA-induced bronchodilatory responses were blocked using inhibitors for NOS and COX enzymes. In summary, we demonstrate that NMDA-R subunit expression and receptor-mediated Ca^{2+} mobilization in HASM cells is critically regulated by TNF, while NMDA-R activation causes dilation in airways when exposed to pro-inflammatory cytokine milieu. These findings improve our understanding about ionotrophic glutamate NMDA-R and their contribution to airway hyperreactivity in asthma.

5.2.0 INTRODUCTION

Asthma is a chronic inflammatory airway disorder, where human airway smooth muscle (HASM) remodeling contributes phenomenally to reversible bronchial obstruction, exaggerated bronchospasms and airway hyperresponsiveness (AHR) [105]. Phenotypic changes in HASM are orchestrated by various pro-inflammatory cytokines released into the airway milieu [60, 288, 289], prominent among which is tumor necrosis factor (TNF) a pleiotropic cytokine produced by various immune and structural cells in the lung. While TNF causes release of several inflammatory mediators (RANTES, TSLP, IL-5, IL-8, eotaxin-1, IL-6 and GM-CSF) [44, 60], it deregulates intracellular calcium ($[Ca^{2+}]_i$) signaling mechanisms and promotes hypercontractile phenotype of HASM to agonists like bradykinin and acetylcholine to elicit pathological complications associated with asthma

[59, 290, 291]. Previously, we showed that HASM cells exhibit a unique receptor-operated calcium entry (ROCE) mechanism through a family of functional glutamate receptors called *N*-methyl-*D*-aspartate receptors (NMDA-R). Originally described in the central nervous system (CNS), NMDA-R are ligand-gated multi-subunit calcium channels activated by simultaneous binding of glutamate (agonist) and glycine (co-agonist) [292]. We demonstrated that NMDA-R activation in HASM cells significantly increases intracellular calcium ($[Ca^{2+}]_i$) mobilization, and mediates airway myocyte contraction both *in vitro* and *ex vivo*. Literature suggests involvement of glutamatergic signaling through NMDA-R activation in the development of airway hyperreactivity and promoting lung inflammation, albeit only in animal models [256, 293]. Considering NMDA-R regulate intracellular Ca^{2+} flow and contractility in HASM cells, we proceeded to explore mechanisms underlying regulation of NMDA-R expression and function in airway myocytes, and their potential involvement in the pathophysiology of asthma.

In neuroinflammatory disorders, increased TNF inside central nervous system (CNS) promotes glutamate-mediated neuronal cytotoxicity and increases surface expression of NR1, an obligatory subunit of NMDA-R complex [294]. Additionally, TNF enhances NMDA-R trafficking to the lipid rafts [295], increases NMDA-R-mediated calcium permeability [294, 295] differentially regulates NMDA-R subunit expression in mixed glial cultures and promotes autocrine release of glutamate from activated microglial cultures [296, 297]. Inside the airways, dysregulated Ca^{2+} homeostasis together with changes in intracellular calcium mobilization and storage processes in HASM cells are suggested to be key mechanisms underlying TNF-derived AHR and subsequent airway

hypercontractility [59, 124, 298, 299]. Also, elevated TNF levels reported in the bronchoalveolar lavage (BAL) fluid from mild and severe asthmatics correlate with their disease severity [300], Considering TNF-dependent modulation of HASM function as an important regulatory mechanism underlying AHR in asthma, we tested the hypothesis that increased TNF presence will modulate NMDA-R subunit expression, potentiates NMDA-R mediated $[Ca^{2+}]_i$ influxes, and contributes to contractile responses in HASM cells. In this study, using cultured airway myocytes, we profile NMDA-R subunit expression under basal and TNF-stimulated conditions by qPCR, immunoblotting and immunocytochemistry. We further assess the TNF's effect on NMDA-R mediated functional responses to specific agonist, NMDA in the presence and absence of NMDA-R antagonist D-AP5 by measuring – intracellular Ca2+ flux in Fura-2-loaded HASM cells and airway responses using murine thin cut lung slices (TCLS). We also assessed role of NMDA-R activation in airway pathogenesis using a murine airway inflammation model of asthma.

5.3.0 RESULTS

5.3.1 TNF differentially regulates NR1 and NR3B subunit mRNA expression in HASM cells in a dose-dependent manner

NMDA-R are multimeric subunit assemblies that combine together to form a functional receptor complex. To date, 7 receptor subunits have been identified in humans, which are classified as obligatory (NR1), regulatory (NR2A, 2B, 2C and 2D) and inhibitory (NR3A and NR3B) on the basis of their individual function. We earlier reported that

cultured HASM cells express mRNA for multiple NMDA-R subunits. To profile the effect of TNF on NMDA-R subunit expression, we first determined the optimal physiological range of TNF. *In vitro* cultured HASM cells were treated with increasing concentrations of TNF (0.1, 1, 10 and 100ng/mL) for 48h and RNA samples were obtained for qPCR analysis using subunit specific primers. We chose to determine the expression of NR1 and NR3B subunits in our RNA samples. While presence of obligatory NR1 subunit is essential for formation of a functional complex, NR3B is the most abundantly expressed NMDA-R subunit in HASM cells. We revealed that expression of obligatory NR1 and inhibitory NR3B subunits was differentially modified by TNF in a concentration-dependent manner (figure 5.1A and 5.1B, n=3). Compared to unstimulated controls, 10 and 100ng/mL TNF stimulation significantly increased NR1 mRNA expression. An increasing, yet insignificant, trend was observed with 0.1 and 1ng/mL TNF (fig 1A). NR3B mRNA abundance decreased significantly even with low doses of 0.1 and 1ng/mL TNF; while maximal decrease was observed with 10ng/mL and 100ng/mL concentrations (fig 5.1B). Based on these observations, 10ng/mL was determined as working TNF concentration to be used for our subsequent studies.

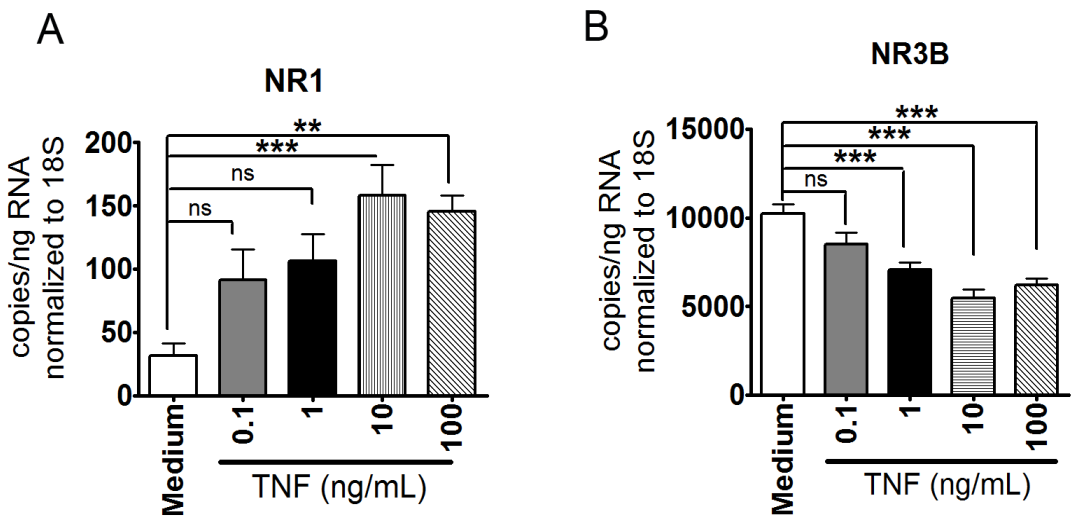
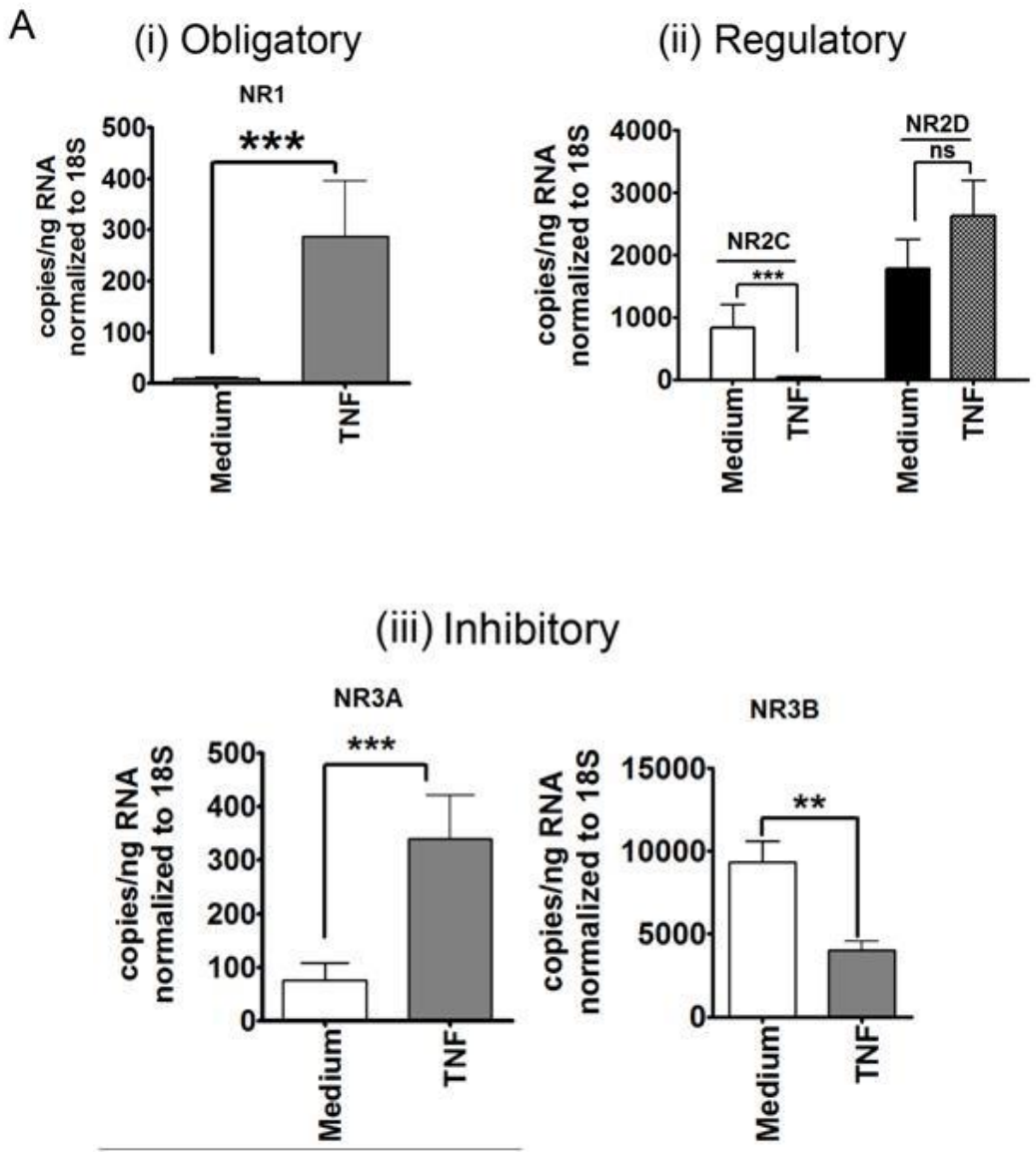


Figure 5.1: *TNF differentially regulates NR1 and NR3B mRNA expression in HASM cells in a dose-dependent manner* – Quantitation of NR1 and NR3B subunits was performed by qPCR using subunit specific primers on samples obtained from cultured HASM cells treated with increasing concentrations (0 – 100ng/mL) of TNF for 48h. Abundance is expressed as copies/ng RNA relative to 18S rRNA. (A) Histogram summarizing NR1 mRNA abundance in confluent HASM cultures maintained in serum-free conditions and treated with 0.1ng/mL, 1ng/mL, 10ng/mL and 100ng/mL TNF. (B) Histogram showing NR3B transcript abundance in TNF-treated HASM cultures. Data represent mean $\pm$ SEM of 3 different experiments performed in triplicates under same experimental conditions. Data was analysed by One-way ANOVA with Bonferroni's multiple comparison tests (***P<0.001, **P<0.01, ns – non significant compared to untreated control).

5.3.2 TNF specifically modifies NMDA-R subunit mRNA expression in HASM Cells in a time-dependent manner

A functional tetrameric NMDA-R complex requires assembly of 2 obligatory NR1 subunits, with NR2 and/or NR3 subtypes. We investigated temporal effect of TNF on mRNA expression of remaining NMDA-R subunits by qPCR using subunit-specific primers in samples obtained from cultured HASM cells stimulated with 10ng/mL TNF for up to 48h. Compared to control samples unstimulated with any cytokine, TNF exposure differentially regulated mRNA expression of various NMDA-R subunits (fig 5.2A and

5.2B, n=6). Post 48h TNF stimulation, HASM cells displayed ~300fold increase in obligatory *NR1* subunit transcript levels (fig 5.2A, i). Among regulatory subunits, *NR2C* transcript levels decreased substantially and *NR2D* expression remained unchanged (fig 5.2A, ii). We found no evidence of NR2A and NR2B subnit expression in our samples. Similarly, inhibitory subunits *NR3A* and *NR3B* displayed a contrasting expression profile: NR3A expression increased considerably NR3B transcript abundance decreased by ~50% (fig 5.2A, iii). Further, HASM cells exposed to TNF displayed a time-dependent increase in NR1 mRNA expression over 48h treatment period (fig 5.2B, i); while NR2C and NR3B mRNA profile decreased over time (fig 5.2B, ii and iii). *NR3A* expression demonstrated a transient increase at 6h and 48h following TNF stimulation but returned to basal values at other time points. We are yet to identify the significance of this varied expression pattern of individual subunits following TNF stimulation. Interestingly, our experimental findings suggest transcription of NMDA-R subunits in HASM cells is under specific regulation of TNF as we did not observe any change in subunit mRNA expression when cells were exposed to either IFNγ or IL-4, under our experimental conditions (data not shown). Taken together, we demonstrate that TNF, which is considered as a classical prototype cytokine representing inflammation, plays an important role in the transcriptional regulation of multiple NMDA-R subunits.


A
(i) Obligatory
NR1
copies/ng RNA normalized to 18S
500
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300
200
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TNF
(ii) Regulatory
copies/ng RNA normalized to 18S
4000
3000
2000
1000
0
NR2C
***
NR2D
ns
Medium
TNF
Medium
TNF
(iii) Inhibitory
NR3A
copies/ng RNA normalized to 18S
500
400
300
200
100
0
***
Medium
TNF
NR3B
copies/ng RNA normalized to 18S
15000
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Medium
TNF

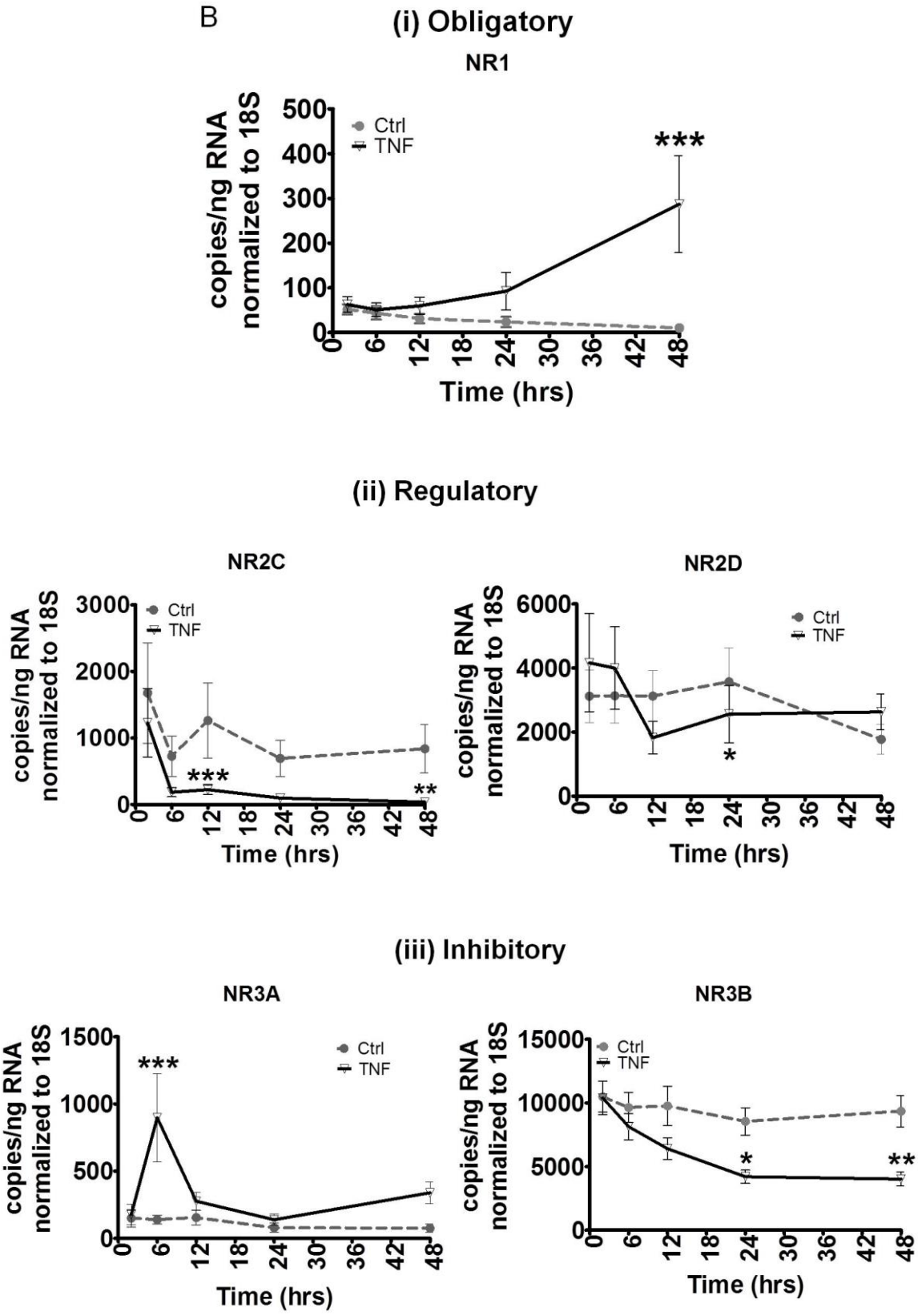
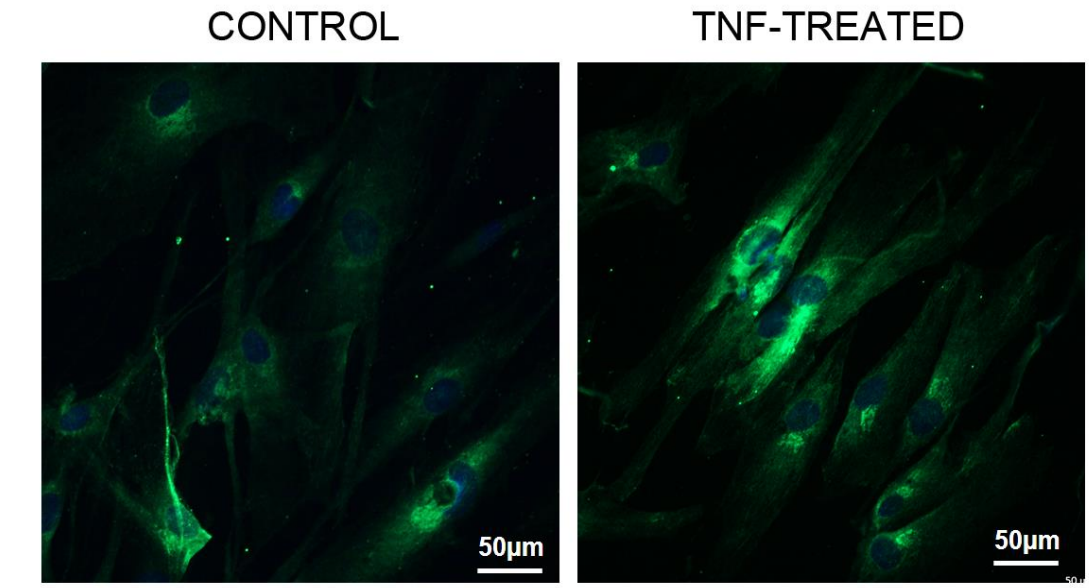

B
(i) Obligatory
NR1
copies/ng RNA normalized to 18S
Ctrl
TNF
***
Time (hrs)
(ii) Regulatory
NR2C
NR2D
(iii) Inhibitory
NR3A
NR3B

Figure 5.2: *TNF specifically modifies mRNA expression of NMDA-R subunits in HASM cells in a time-dependent manner* – mRNA quantitation by qPCR was performed for NMDA-R subunits in samples from confluent HASM cultures treated with 10ng/mL TNF in serum-deprived conditions. Primers designed against specifically against different NMDA-R subunits were used to determine the quantitative expression of these genes. (A) Abundance of mRNA for respective obligatory (NR1), regulatory (NR2C and NR2D) and inhibitory (NR3A and NR3B) NMDA-R subunits in confluent HASM cultures maintained in serum-free medium and TNF-treated conditions for 48h. Data was analysed by unpaired Student's *t*-test. (**P<0.01, ***P<0.001 and ns (not significant) compared to control) (B) Time-course for mRNA abundance of obligatory (NR1), regulatory (NR2C and NR2D) and inhibitory (NR3A and NR3B) subunits in control and TNF-treated HASM cultures in serum-free conditions. Data was analyzed by two-way ANOVA with Bonferroni multiple comparison post-hoc tests (***P<0.001, **P<0.01, *P<0.05 compared to control). Data represents mean values from 6 different independent experiments performed in triplicates under similar experimental conditions. Error bars are shown as mean $\pm$ SE.

5.3.3 TNF induces NR1 protein expression in HASM cells

To further substantiate our qPCR observations on TNF's role in regulating NMDA-R expression on HASM cells, we determined NR1 protein expression by immunofluorescence and Western blotting (Fig 5.3A and B). HASM cells were cultured *in vitro*, stimulated with TNF, IFNγ or IL-4 respectively (10ng/mL) for 48h and stained for NR1 protein. While NR1 expression was detected in control HASM cells untreated with any cytokine, TNF-treated cells exhibited a significant increase in NR1 fluorescence, which correlated with our qPCR observations (fig 5.3A, n=3). However, we did not observe any change in fluorescence intensity in cells stimulated with either IFNγ or IL-4 (data not shown). Further, we confirmed our findings by immunoblotting using cell lysates from 1 immortalized and 3 primary human ASM cell lines. When exposed to 10ng/mL TNF, we observed a non-quantitative trend indicating increased NR1 expression. An identical blot was probed for GAPDH expression to show equivalent protein loading (fig

5.3B, n=4). Detection of other NMDA-R subunits was not possible due to absence of specific antibodies.



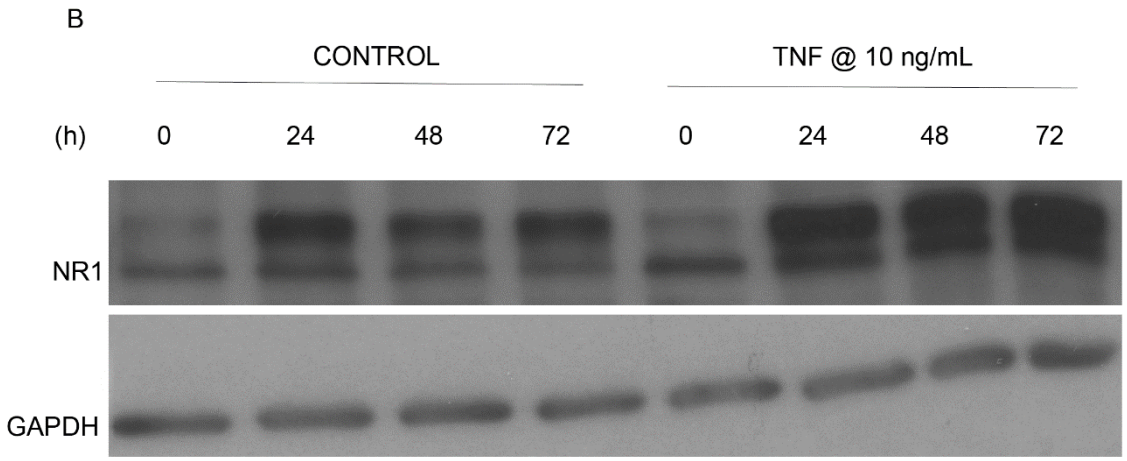


Figure 5.3: *TNF induces NR1 protein expression on HASM cells – In vitro* cultured HASM cells were treated with 10ng/mL TNF and analyzed for NR1 protein expression by immunofluorescence (A) and western blot (B). Representative IF images (confocal) demonstrate an increase in NR1

expression in TNF-treated cells compared to control untreated myocytes (A, (i) and (ii)). Nucleus was stained with DAPI and NR1 with AF-488. Immunofluorescence images represent 3 independent experiments done in similar conditions. To determine TNF effect on NR1 expression by western blot, we used protein lysates from 4 different HASM cell lines (1 immortalized and 3 primary). This is a representative figure (B) showing a time-dependent increase in protein expression of ~120kDa obligatory NR1 subunit. Whole mouse brain lysate was used as a positive control (data not shown).

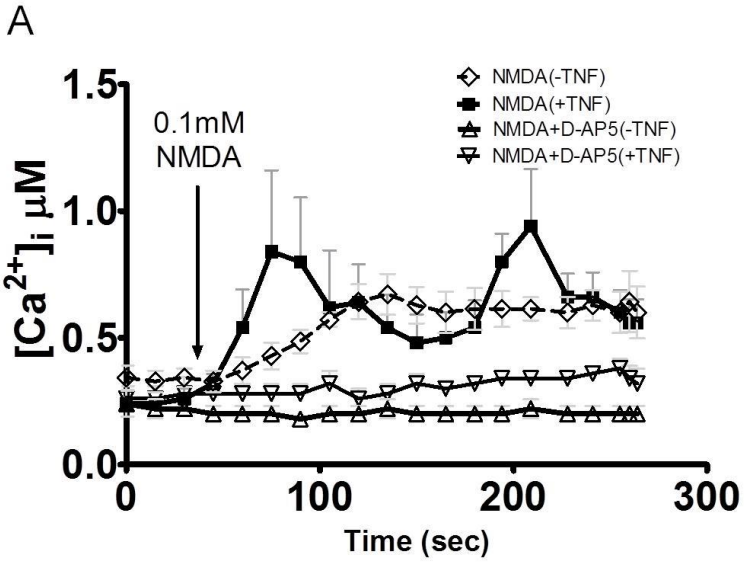
5.3.4 NMDA-R-mediated intracellular Ca^{2+} responses are induced in TNF-treated HASM Cells

Intracellular calcium mobilization is central determinant of ASM-mediated pulmonary responses including airway contraction and relaxation [298]. Existing research suggests TNF modulates ASM function and contributes to hyperresponsiveness in airways by increasing Ca^{2+} sensitivity and altering Ca^{2+}-dependent force generation within HASM cells [59]. We have previously shown that NMDA-R activation on HASM cells mobilizes free Ca^{2+} from extracellular spaces into the cell. To further examine TNF's impact in the regulation of NMDA-R function, we used real-time Ca^{2+} imaging and assessed receptor-mediated $[Ca^{2+}]_i$ in TNF-treated and Fura-2-loaded HASM cells. For these experiments, weused NMDA, a pharmacological glutamate analogue that binds specifically to NMDA-R and activates the receptor. Compared to control unstimulated cells, TNF-treated cells exhibited a different calcium mobilization pattern with NMDA (0.1mM) stimulation (fig 5.4A; n=6). Following agonist stimulation, Ca^{2+} influx was observed within ~5-10seconds in TNF-treated HASM cells, which also displayed a phasic response pattern with a 2^{nd} peak observed ~160seconds after NMDA-R activation (peak 1 = 0.87µM at 75s; peak 2 = 0.93µM at 209s). On the contrary, control cells showed a sustained NMDA-elicited mean Ca^{2+} response that began slowly at ~100sec after adding agonist to the cells. Peak Ca^{2+}

levels in TNF-treated and untreated HASM cells were not statistically significant (fig 5.4B, n=6), but we observed an increasing trend under our experimental conditions. “Area under the curve” analysis showed a greater area in TNF-treated cells (152.7) in comparison to untreated cells (139.1), which suggests increased $[Ca^{2+}]_i$ flux into the cells upon NMDA-R activation with TNF exposure (fig 5.4C). TNF-induced NMDA-R-mediated $[Ca^{2+}]_i$ responses were abrogated significantly when cells were treated with 0.5mM D-AP5, a receptor specific antagonist.

In our previous study, we showed that HASM cells demostrate an intercellular heterogeniety in temporal Ca^{2+} response pattterns upon NMDA-R activation. Based on the response time and pattern of Ca^{2+} mobilization demonstrated by each individual cell, we classified them into 4 distinct sub-groups: “Early Response”, “Late Response”, “Phasic Response” and “No response”. In our current study, we obseved a redistribution of these response sub-groups when HASM cells were pre-treated with TNF and stimulated with 0.1mM NMDA. Compared to control unstimulated cells, TNF-exposed cells showed a substantial decrease in “No-response” sub-group, which correlated with significant increase in the number of cells mobilizing Ca^{2+} with NMDA stimulation (fig 5.4D, n=6) In TNF-exposed HASM cells, 94.16% cells responded to NMDA stimulation 76.85% in control cells) demonstrating an elevated $[Ca^{2+}]_i$ flux and 5.83% cells did not show any response (23.1% in control group). Amongst the total “responder” population in TNF-treated cells, we observed 36.6% of cultured HASM cells were of “Early Response” sub-group characterized as cells responding immediately (within 5-10s) to NMDA stimulation. We found 32.8% belonged to “Late Response” group, displaying a delayed increase in Ca^{2+}

flux ~60-100s after agonist stimulation,. We also identified 25.2% of all myocytes showing a phasic response profile, where cells presented multiple Ca^{2+} spikes within the time frame of measurment (300s) (fig 5.4E n=6). Together, these results provide evidence that NMDA-R-mediated increase in cytosolic calcium is enhanced when cultured airways are exposed to TNF, which also modulates inherent heterogeneity to NMDA-induced Ca^{2+} mobilization in different sub-groups of HASM cells. These observations suggest an increase in the number of NMDA-R complexes on the membrane surface or a possible re-arrangement of NMDA-R subunits within the receptor complex causing increased Ca^{2+} flux or both. However, we still need to validate our assumptions.



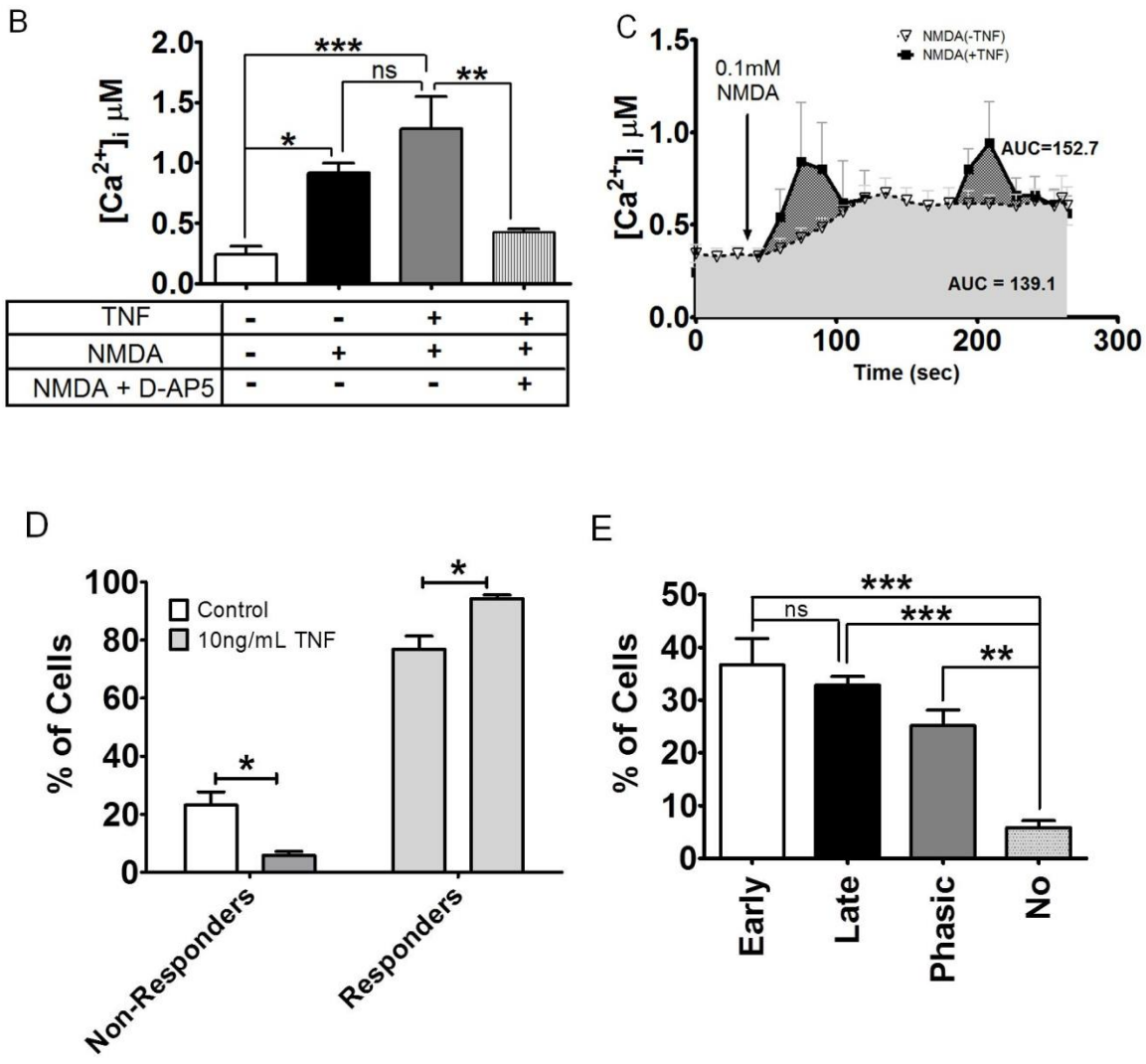


Figure 5.4: *TNF exposure alters NMDA-induced $[Ca^{2+}]_i$ responses in HASM cells* – (A) Representative tracings of calcium using Fura-2 loaded ASM cells. TNF-treated and control HASM cells were stimulated with 0.1mM NMDA in the absence or presence of 0.5mM D-AP5 and changes in intracellular calcium ($[Ca^{2+}]_i$) were recorded. Each tracing is a mean of 50-60 elongated cells from 6 separate experiments (B) Histogram represents the basal (white) and NMDA-induced max $[Ca^{2+}]_i$ response observed when HASM cells were untreated (black), treated with 10ng/mL TNF (chequered) and 0.5mM AP-5 (slashed lines). Data represent mean + SE obtained from 6 independent experiments done in duplicates and analyzed by one-way ANOVA with Bonferroni's multiple comparison test. ***$p<0.001$; *$p<0.05$; ns=non-significant compared to control. **$p<0.01$ compared to TNF-treated cells. (C) Representative tracings showing area under curve (AUC) in control and TNF-treated cells stimulated with 0.1mM NMDA. (D) Histogram representing percentage of cells exhibiting different types of responses in a particular field when stimulated with NMDA. Data represent mean + SE from 6 independent experiments and analyzed by one-way ANOVA with Bonferroni's multiple comparison test. *$p<0.05$ compared to control (E) Histogram

showing distribution (%) of cells exhibiting different temporal responses for Ca^{2+} mobilization in response to 0.1mM NMDA in TNF-treated HASM cells. Tracings from ~30 randomly selected cells in a field were analyzed from 5 independent experiments done in duplicate and individual cell response was categorized into one of four groups: *Early Responses* (white), *Late Responses* (black), *Phasic Responses* (grey) or *No Response* (lined) (n=6, ***p<0.001, **p<0.01 analyzed by one-way ANOVA with Bonferroni's multiple comparison test).

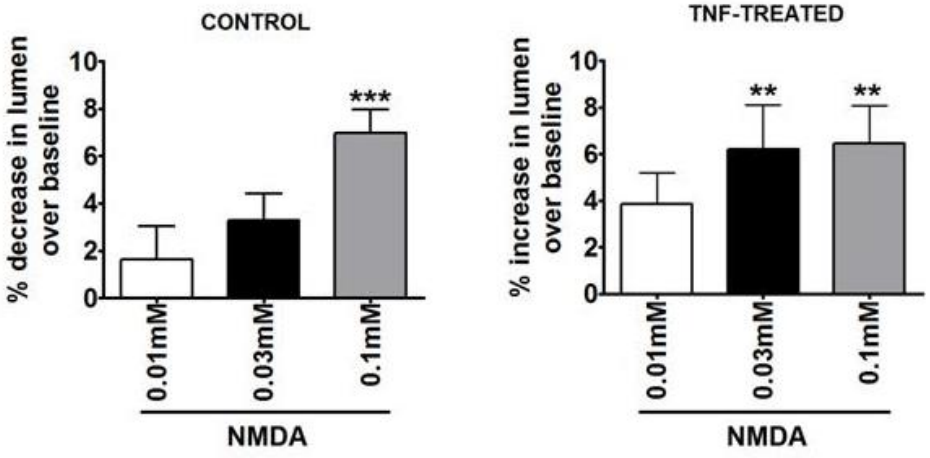
5.3.5 NMDA-R mediates airway relaxation in TNF-treated murine thin-cut lung slices, *ex vivo*

In culture airway myocytes, TNF exposure differentially modulated NMDA-R subunit transcription and translation, including the obligatory NR1 and induced agonist-mediated cytosolic calcium flux. Therefore, we assessed TNF's role in regulating on airway responses due to NMDA-R activationin murine TCLS preparations using time-lapse video microscopy. In these experiments, vibratome sectioned 180µ-sized mouse airway slices were incubated in D-MEM-containing culture medium with or without 10ng/mL TNF for 48h and airway contractions/relaxations were recorded. Previously, we have established that presence of 0.2mM glycine is essential for maximal NMDA-R activation under our experimental conditions. Therefore, 0.2mM glycine was used in all our functional studies. Using a cumulative NMDA dose-response experimental design, we measured airway responses in time-matched control and TNF-treated TCLS. We also determined active airway narrowing to 1µM methacholine (MCh) in all the airways as a positive control and used only those that showed airway narrowing to MCh for our analysis (data not shown). In control airways, we did not observe any significant decrease in airway lumen area from baseline to 0.01 and 0.03mM NMDA, but 0.1mM NMDA significantly narrowed airways by ~7% (fig 5.5A) At the same time, TNF-treated slices exhibited significant increase in airway lumen area from baseline to 0.03mM and 0.1mM NMDA

(fig 5.5A). To confirm airway contractile and relaxation responses were elicited by NMDA-R activation in control and TNF-treated slices respectively, some slices were pre-incubated for 2h with 0.5mM (2R)-amino-5-phosphonopentanoate (D-AP5), a selective NMDA-R antagonist. We found D-AP5 completely blocked NMDA-induced airway responses in both control and TNF-treated slices, which confirms NMDA-R specificity in observed airway behavior (fig 5.5B; n=3).

Further, to determine whether NMDA-induced airway responses in murine TCLS were reliant on Ca^{2+} influx from extracellular spaces, we measured ariway contraction/relaxation in control as well as TNF-treated slices perfused with Ca^{2+}-free HBSS, stimulated with 0.1mM NMDA and airway responses were recorded for 4min. In the absence of extracellular Ca^{2+}, we did not observe any change in airway lumen area with NMDA-R activation.. The same airways were then perfused with Ca^{2+}-containing HBSS for 30min before stimulating them again with 0.1mM NMDA (in the presence of Ca^{2+}) and lumen area increase/decrease was measured. With the replenishment of Ca^{2+} in the buffer, we observed that control airways contracted with NMDA, while TNF-treated airways displayed an increased lumen area over baseline. Finally, the same airways were perfused with Ca^{2+}-HBSS containing 1mM EDTA for another 30min to chelate extracellular calcium in the buffer, stimulated with 0.1mM NMDA and airway response was measured. Again, we observed no NMDA-elicited response in airways when extracellular calcium was chelated both in untreated and TNF-treated lung slices (fig5. 5C; n=3). Together, these data indicate that NMDA-R mediated airway responses in murine TCLS are differentially regulated by TNF, and that presence of extracellular Ca^{2+} is required for this effect.

A


CONTROL
TNF-TREATED
% decrease in lumen over baseline
% increase in lumen over baseline
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2
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***
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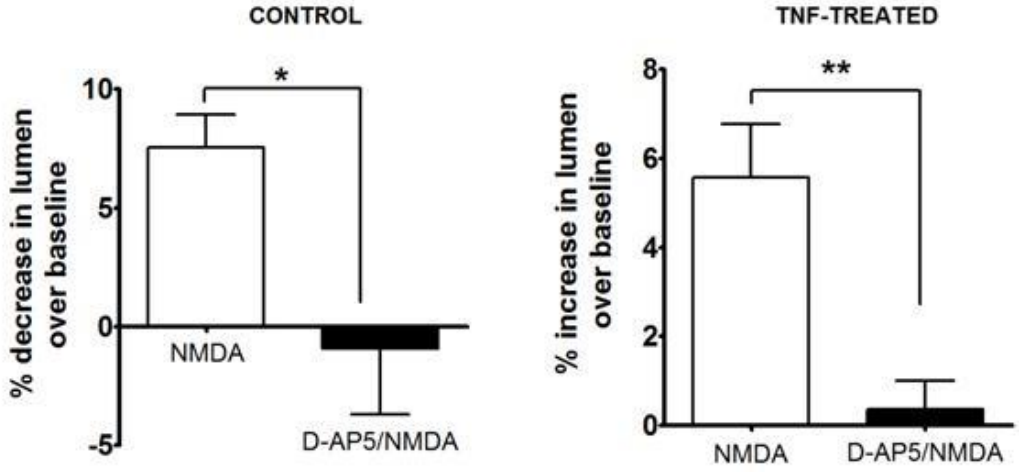
NMDA

B


CONTROL
TNF-TREATED
% decrease in lumen over baseline
% increase in lumen over baseline
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-5
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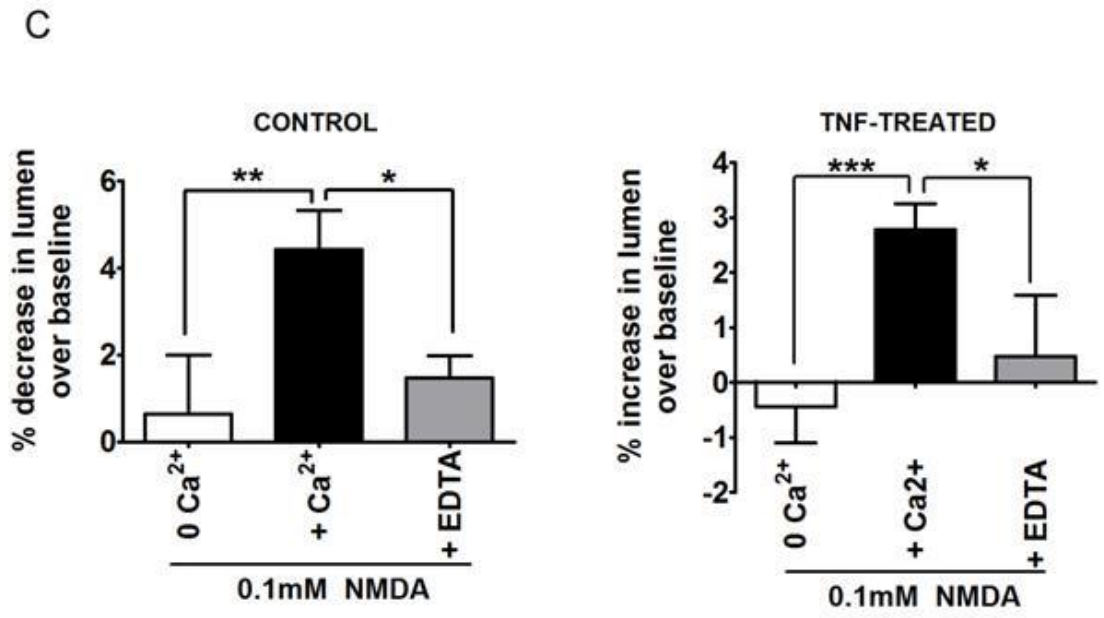


Figure 5.5: *NMDA-R activation induces Ca^{2+}-dependent airway dilation in TNF-treated mouse lung slices* - (A) Histogram showing mean airway responses in time-matched control (a) versus TNF-treated (b) murine lung slices. In control slices, concentration-dependent airway narrowing was observed with 0.01mM (~1.8%), 0.03mM (~3%) and 0.1mM NMDA (~7.5%) stimulation. In TNF-treated slices, 0.01mM NMDA stimulation caused ~4% increase in airway lumen area, and ~6% with 0.03mM and 0.1mM NMDA. Slice was washed for 30min with HBSS allowing airway relaxation to almost baseline. This was followed by stimulation with 1µM MCh allowing airway to shorten by ~18%. Data represent mean ± SE obtained from 12-13 airways from 3 mice and analyzed by one-way ANOVA. ***p<0.001; **p<0.01 compared to control. (B) Histogram summarizing the mean response of NMDA on airway narrowing/dilation measured in control vs TNF-treated TCLS before and after treatment with 0.5mM D-AP5, a NMDA-R antagonist. Data represent mean ± SE obtained from 7 airways from 3 different mice and analyzed by unpaired student *t* test. **p<0.01; *p<0.05 compared to control (C) Histogram showing mean airway narrowing or dilation in TCLS in the presence and absence of extracellular Ca^{2+}. In an airway slice, response to 0.1mM NMDA was first recorded in Ca^{2+}-free HBSS, washed for 30min with Ca^{2+}-containing HBSS and NMDA response was recorded again in the presence of Ca^{2+}. Slice was washed once again with HBSS containing 1mM EDTA for 30min and NMDA-mediated response was recorded in the same buffer. Absence of Ca^{2+} in the buffer completely abrogated NMDA-R-mediated airway narrowing/dilation in control and TNF-exposed slices respectively. Data represent mean ± SEM obtained from 6 airways from 3 individual mice and was analyzed by one-way ANOVA with Bonferroni multiple comparison test (***P<0.001, **P<0.01, *P<0.05).

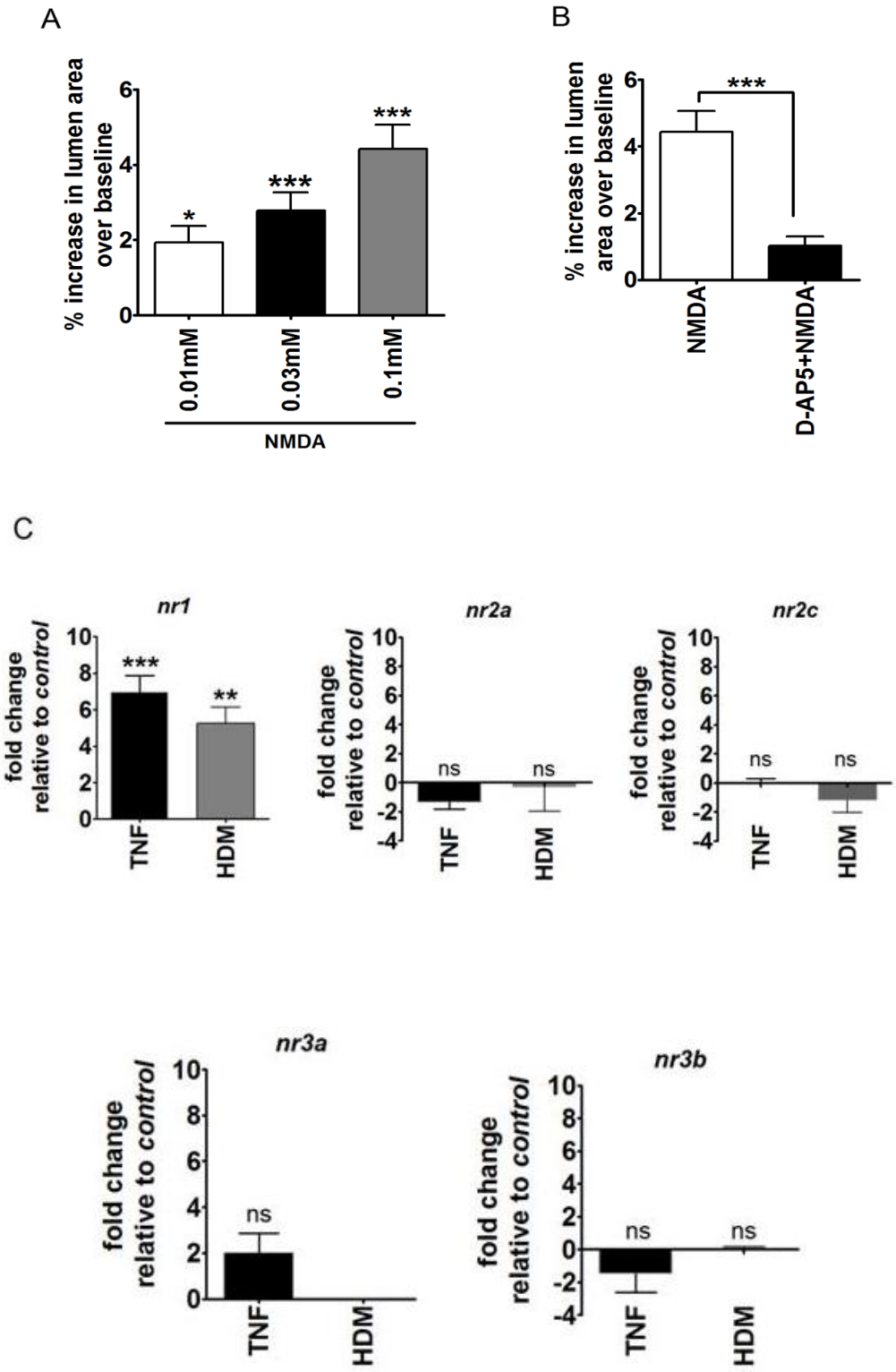
5.3.6 NMDA-R stimulation causes airway dilation in HDM-exposed murineTCLS, *ex vivo*

We next determined whether altered airway responses to NMDA stimulation following TNF pre-treatment contributes to increased AHR using a HDM-sensitized mouse model of acute allergic inflammation. Periodic delivery of HDM intranasally into mice is one of the most widely used *in vivo* models to study airway inflammation and AHR occuring in allergic airway diseases such as asthma [301]. 48h following final HDM challenge, mouse lungs were agarose-inflated, sectioned into 180µ-sized TCLS, incubated for 24h in sterile D-MEM and NMDA-mediated airway responses were recorded using an isolated perfusion system. When stimulated with increasing concentrations of NMDA (0.01 – 0.1mM), HDM-exposed TCLS exhibited significant dose-dependent increase in lumen area in airways suggesting NMDA-R activation dilates airways in mice during allergic inflammation. Airways showed 1.9% increase in lumen area when perfused with 0.01mM NMDA, airway dilation increased to 2.78% with 0.03mM and 4.42% with 0.1mM NMDA stimulation (Fig 5.6A, n=6). NMDA-induced responses were signficantly blocked when airways were incubated with 0.5mM D-AP5, thereby confirming an NMDA-R specific effect (fig 5.6B).

In the nervous system, NMDA-Rs are known to elicit alternative cellular responses dependent primarily on the subunit configuration on the membrane. To delineate bronchodilatory responses due to NMDA-R activation in murine TCLS exposed to inflammatory stimuli, we performed qPCR analysis for NMDA-R subunits in cDNA

samples form control, TNF-treated and HDM-exposed mouse lung slices. We found that murine airway slices have relative mRNA abundance for *nr1, nr2a, nr2c, nr3a* and *nr3b* genes, but were unable to identify the presence of *nr2b* and *nr2d* genes. Compared to control samples, we observed a significant increase in *nr1* transcript expression, both in TNF and HDM-exposed lung slice specimens, which was in accordance with our findings in HASM cells. While mRNA expression of other subunits showed a decrease compared to control, none of them were statistical significant. (Fig 5.6C, TCLS from 3-5 mice were used for this analysis).

To further understand NMDA-R's role under physiological and pathological conditions, we performed lung function analysis in naive and HDM-exposed mice using FlexiVent, a small animal lung ventilator system. Airway resistance (R_{aw}), tissue resistance (G) and tissue elastance (H) were determined in these animals using increasing doses of aerosolized NMDA (0.1mM – 100mM). Response to 3mg/mL MCh was used as our reference control. Contrary to the results we obtained using murine lung slices, NMDA exposure did not demonstrate measurable changes in any of the lung function parameters both in control and HDM-treated mice (Fig 5.6D (i – iii), n=6). While these observations suggest NMDA does not have direct effect on airway physiology, it does not rule out the possibility of NMDA-R indirectly influencing the function of methacholine receptors and other contractile agonists as mentioned in some other studies. However, such assumptions require further investigation.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nr1
fold change relative to control
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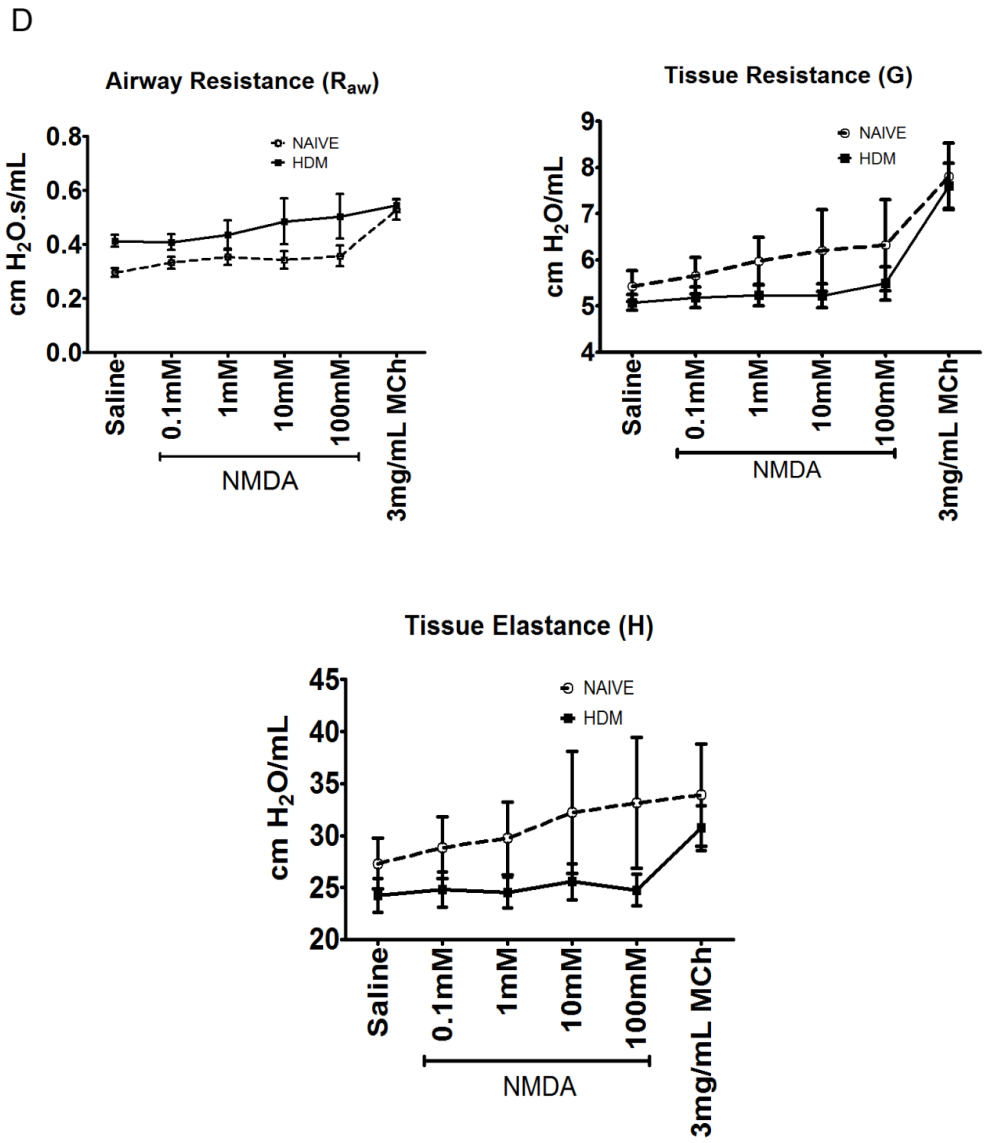


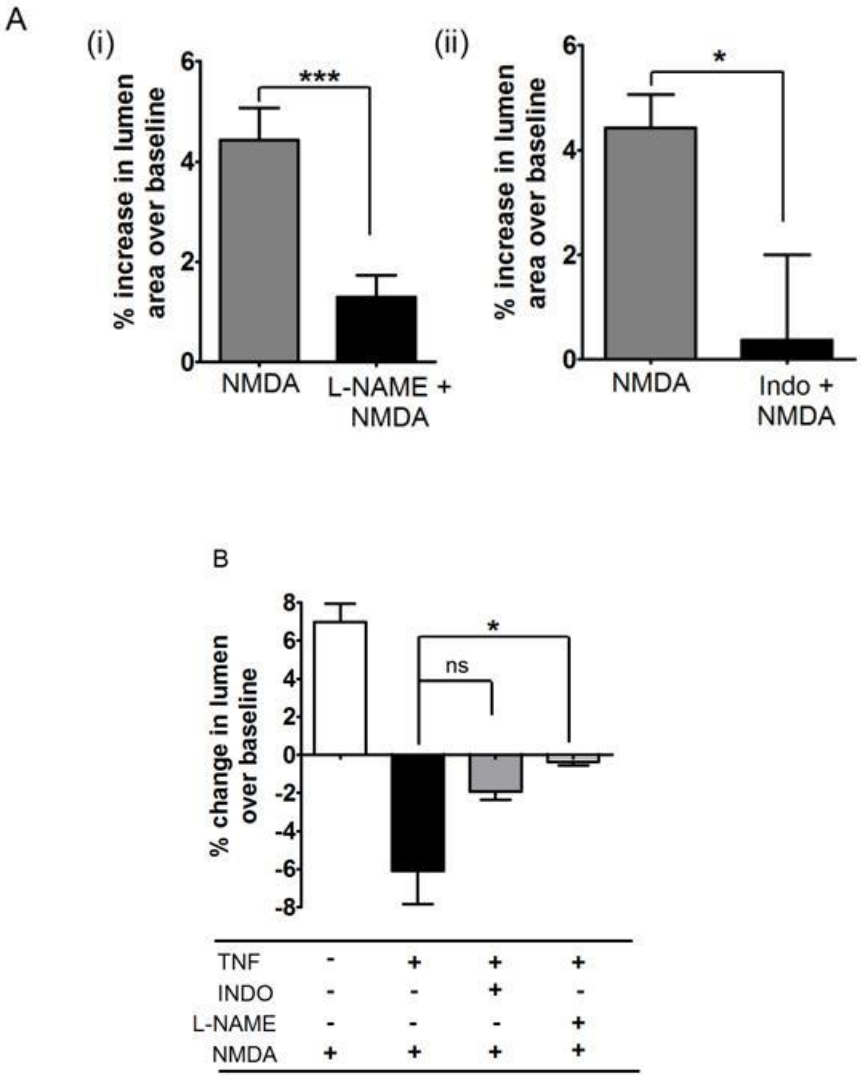
Figure 5.6: *NMDA-induced airway dilation in HDM-exposed mouse lung slices* – **(A)** Histogram showing NMDA concentration-dependent increase in airway lumen area (10µM – 100µM) in HDM-exposed lung slices. Data represent the mean ± SE from 14 airways obtained from 6 individual mice and analyzed by two-way ANOVA ***p<0.001; *p<0.05 compared to baseline. **(B)** Histogram of mean airway dilation in HDM-exposed and 0.5mM D-AP5 pre-treated lung slices and stimulated with 0.1mM NMDA Data represent the mean ± SE from 13 airways obtained from 6 individual mice and analyzed by unpaired student *t* test ***p<0.001; compared to baseline. (**(C)** qPCR was performed on mRNA isolated from untreated, TNF-treated and HDM-exposed mouse lung slices using specific primers for mouse *nr1, nr2a, nr2c, nr3a* and *nr3b* genes. Data represent the mean ± SE from 3-5 mice and analyzed by unpaired student *t* test. ***p<0.001; ns = non-significant compared to control. **(D)** Lung function in HDM-exposed mice compared to naive mice was assessed by FlexiVent. Measurement of airway mechanics was done after inhalation of nebulized saline, followed by increasing concentrations of NMDA (100µM – 100mM). NMDA

dose-response curve for (i) airway resistance (Raw) (ii) tissue resistance (G) and (iii) tissue elastance (H) were plotted for each group. 3mg/mL MCh dose was used as our internal control. No statistical difference was observed between naive and HDM-challenged mice. Data represent mean ± SEM and was analysed by One-way ANOVA with Bonferroni multiple comparison tests (***P<0.001, **P<0.01, *P<0.05).

5.3.7 NMDA-induced airway dilation in murine lung slices is mediated via iNOS and COX-2 dependent mechanisms

In studies done by Said *et al* and others, NMDA-R-mediated effects on lungs and airways were blocked effectively using inhibitors of inducible nitric oxide synthase (iNOS) and involved COX-2 activation [252, 302, 303]. Also, NMDA-R activation causes synthesis of nitric oxide (NO) [304]. Interestingly, both NO release and COX-2 activation are implicated as important ASM relaxation mechanisms [140, 305, 306]. We determined whether NMDA-R mediated airway dilatory responses during inflammation involve NO and COX. For this, airway slices were pre-treated for 1h with 0.1mM L-NAME (a non-specific NOS inhibitor) or 0.01mM indomethacin (a non-specific COX inhibitor) and airway responses were recorded after stimulation with 0.1mM NMDA (Fig 5.7A (i and ii), 13 airways from 5 individual mice). Under our experimental conditions, we observed that L-NAME and indomethacin significantly abrogating NMDA-induced increase in lumen area, both in HDM-exposed and TNF-treated airway slices (Fig 5.7B, 6 airways from 3 individual mice,). This suggests NMDA-mediated bronchodilation in inflamed airway slices occurs via NOS and COX activation. To further understand the underlying mechanisms, we used qPCR to determine the effect of TNF on the mRNA expression of *inos*, *cox-1* and *cox-2* genes. We used mouse TATA-binding protein (*tbp)* as our reference control because of its stable expression across different tissues. Surprisingly, none of the

enzymes demonstrated any significant change in their transcript relative abundance, both in TNF-treated and HDM-exposed lung slices compared to untreated controls suggesting the possibility of differential induction of *nos* and *cox* genes in mouse airways by NMDA-R rather than change in gene expression levels (fig 5.7C (i – iii), n=5).



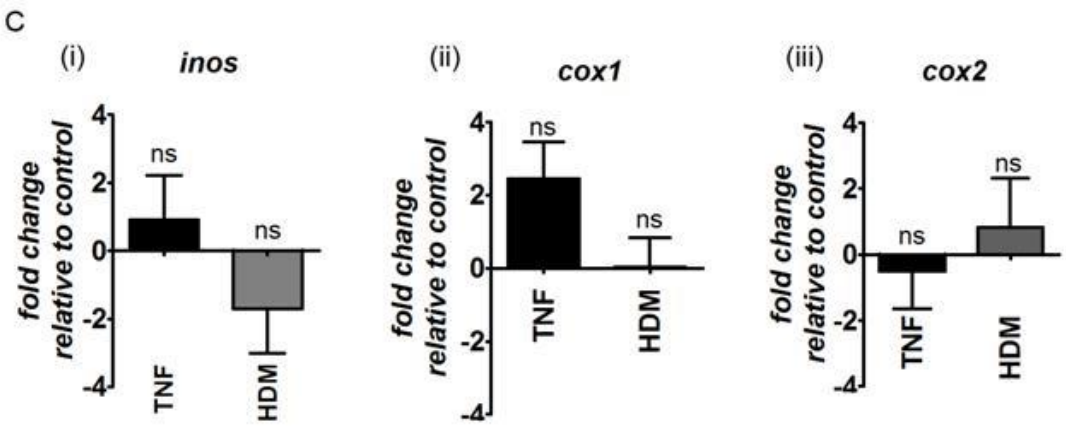


Figure 5.7: *Effect of NOS and COX on NMDA-induced airway dilation in HDM-exposed mouse lung slices* – (A) Histogram showing mean airway responses in HDM-exposed lung slices treated with 0.1mM L-NAME (i) or 0.01mM indomethacin (ii) and stimulated with 0.1mM NMDA. Data represent the mean ± SE from 13 airways obtained from 5 different mice and analyzed by unpaired student *t* test ***p<0.001; *p<0.05 compared to 0.1mM NMDA (B) Histogram showing mean airway responses control and TNF-treated lung slices treated with 0.1mM L-NAME (i) or 0.01mM indomethacin (ii) and stimulated with 0.1mM NMDA. Data represent the mean ± SE obtained 6 airways from 3 mice and analyzed by one-way ANOVA *p<0.05; ns = non-significant compared to TNF-treated slices. Positive values represent airway contraction and negative values indicate airway relaxation **(C)** qPCR was performed on mRNA isolated from untreated, TNF-treated and HDM-exposed murine lung slices using specific primers for *inos*, *cox1* and *cox2* genes (N=5). Data represent mean ± SEM and was analysed by unpaired student *t* test with Bonferroni multiple comparison tests (ns = non-significant).

5.4.0 DISCUSSION

Receptor-operated cation channels (ROCCs) expressed on HASM cells and their contributions to changes in $[Ca^{2+}]_i$ are long considered important determinants of ASM responses including contraction and relaxation [125]. In chronic airway conditions such as asthma, these responses are critically regulated by the presence of various pro-inflammatory mediators inside the airway microenvironment [110]. Previously, we were the first to report the presence of functional NMDA-R as a novel ROCC in human ASM

cells promoting airway contractility. In our present study, we examined the influence of pro-inflammatory cytokine TNF on NMDA-R subunit expression and receptor-mediated $[Ca^{2+}]_i$ signaling in human ASM cells. Further, we investigated the contribution of NMDA-R activation to airway responsiveness under chronic inflammatory pathologies using a HDM-sensitized and challenged murine model of airway inflammation. For the first time, we have shown that NMDA-R expression and functionality in HASM cells is definitively regulated by TNF. Our results demonstrate pro-inflammatory cytokine TNF differentially modulates NMDA-R subunit expression in HASM cells, alters NMDA-induced intracellular calcium mobilization and causes airway dilation in murine TCLS. In addition, we report that TNF-dependent modulation of NMDA-R-mediated murine airway responses are mediated via an NOS and COX-dependent mechanism.

Most studies done to elucidate the presence and functional significance of ionotrophic NMDA-R in different lung components were performed primarily in animal models or tumor cells [244, 253, 255]. Further, there exists limited evidence on mechanisms influencing receptor regulation in lungs and airways. Literature suggests inflammation, in particular TNF, induces differential regulation NMDA-R subunit expression occurs in various neuronal and non-neuronal compartments [294]. Concomitantly, we also observed that HASM cells, when pre-treated with TNF, show increased expression of obligatory NR1, in a time-dependent and dose-dependent manner both at the mRNA and protein level. At the same time, TNF persistenly decreased transcript levels of regulatory NR2C and inhibitory NR3B subunits, except NR3A and NR2D.

Based on mRNA expression data, it can be hypothesized that TNF exposure leads to subunit arrangement, particularly amongst the regulatory and inhibitory subunits, at least in some percentage of HASM cells. Plasticity in NMDA-R subunit composition and distribution depending on the stage of development and tissue microenvironment is well-defined in neurons [295]. While our observations from $[Ca^{2+}]_i$ experiments tend to support such a scenario in airway myocytes, future studies are required to directly correlate expression of multiple NMDA-R subunits with different HASM cell populations, which can be technically challenging owing to lack of appropriate subunit-specific antibodies. Heterogeneity in NMDA-R subunit expression with distinct pharmacological properties translates to variations in receptor function eventually leading to varied susceptibility to glutamate toxicity in neurons [307]. For instance, receptors comprised of NR2A and NR2B cause larger cell death compared to NMDA-Rs with NR2C or 2D subunits, which is attributed to differential mobilization of $[Ca^{2+}]_i$ [307]. We observed similar alterations in NMDA-induced calcium mobilization in HASM cells pre-treated with TNF. While TNF exposure did not change the magnitude or peak rise in mean calcium levels, HASM cells exhibited a noticeable change in calcium flux patterns with NMDA stimulation. At the same time, there was considerable increase in total calcium flow with TNF treatment when AUC (area under the curve) was analyzed, while number of cells not responding to NMDA stimulation decreased significantly. Taken together, these observations suggest a significant increase in NMDA-responsive HASM cells by TNF occuring due to increased expression of NR1-containing receptor. It is significant to note that a similar distribution of calcium response patterns at a single-cell level has been reported in T lymphocytes, which alters as cells differentiate from naive to memory phenotype [308]. It is known that

agonist-elicited Ca^{2+} responses in ASM cells are dynamically regulated in a spatio-temporal pattern and display intercellular heterogeneity [122, 284]. One suggested reason for such a behavior is differential distribution of receptors on the cell membrane. We believe NMDA-Rs on ASM cells exhibit a similar phenomenon. Further, TNF regulates changes in NMDA-elicited $[Ca^{2+}]_i$ responses by altering receptor distribution, which can be blocked by D-AP5, an NMDA-R antagonist. It will be interesting to see whether other ROCCs exhibit a similar $[Ca^{2+}]_i$ flux behavior. Even though calcium-dependent excitation-contraction coupling in HASM cells has been extensively investigated, we still are far from understanding Ca^{2+}-handling processes under pathological conditions. It is believed that frequency of generated Ca^{2+} waves rather than peak amplitude determines the force during a contractile response[105]. From our experimental findings, we can hypothesize that proinflammatory cytokines such as TNF augment agonist-elicited Ca^{2+} wave frequencies and promotes increased calcium entry via altering the membrane distribution and receptor localization of ROCCs across heterogenous ASM bundles. Unfortunately, we were unable to determine receptor subtype configuration in TNF-treated and untreated HASM cells due to lack of appropriate antibodies against NR2 and NR3 subunits and our inability to segregate HASM cell populations based on their calcium influx properties.

The presence of glutamatergic signaling in lungs and regulation of airway responsiveness by NMDA-R in respiratory pathologies such as asthma remains controversial and inconclusive. Studies that attempted to elucidate the role of these receptors were performed using various experimental airway inflammation models. In these animals, NMDA was administered intraperitoneally and NMDA-specific airway

responses were determined indirectly as a measure of reactivity to contractile agonists such as acetylcholine and histamine. However, these experiments do not provide any information regarding direct involvement of NMDA-R in airways, particularly in ASM cells. In our studies, we wanted to address this discrepancy and identify the direct influence of NMDA-R in ASM-mediated pathological airway responsiveness *ex vivo* using agarose-inflated murine lung slices. We demonstrated that NMDA stimulation induced an increase in airway lumen area in a dose-dependent manner, both under TNF-treated and HDM-exposed conditions suggesting NMDA-R activation acts as a bronchodilator under inflammatory conditions. Inhibition of these responses by D-AP5 further ascertained NMDA-R specificity in regulating these responses. Whether these responses are clinically relevant in humans remains unknown. Also, we determined that NMDA-induced dilation observed in TNF-treated and HDM-challenged airway slices were Ca^{2+}- dependent. In the absence of extracellular Ca^{2+} or in the presence of Ca^{2+} - chelator EDTA, NMDA was unable to dilate airway lumen in TNF-treated slices. Additionally, compelling evidence from our studies done using HASM cells suggests that NMDA-R-mediated airway responses are Ca^{2+} driven specifically by ASM tissue.

Using non-specific inhibitors, we demonstrated that NOS and COX-dependent mechanisms are involved in mediating NMDA-induced bronchodilation in inflamed mouse lung slices. Taking into consideration available evidence suggesting iNOS and COX-2 are major mediators of dilatory response in airways, we believe occurence of a similar model in our experimental conditions. Wu *et al* have shown that TNF induces iNOS and COX-2 expression in tracheal SMCs [309]. While iNOS expression increased NO formation, TNF-

inducible COX-2 activation caused prostaglandin E2 (PGE2) secretion in these cells. Similar observations were made in other pulmonary cell types such as epithelial and endothelial cells [310, 311]. Available research data suggests activation of iNOS causes NO synthesis, which activates COX-2 leading to synthesis and release of PGE2 and airway relaxation [312]. When we consider that no changes were seen in mRNA abundance of *inos*, *cox-1* and *cox-2*, the potential contribution of endothelial NOS (eNOS) in NO production and subsequent airway dilation observed in our murine lung slices cannot be ignored. Inside the lungs, eNOS is expressed by multiple cell types including ASMCs and its cellular regulation is largely dependent on increased calcium levels [313, 314]. Studies have shown that NMDA-mediated vasodilatory responses are eNOS-dependent and involve intracellular sequestration of Ca^{2+} [315, 316]. On a related note, we showed that TNF exposure altered NMDA-R-mediated Ca^{2+} mobilization patterns in HASM cells that might cause eNOS activation and release of NO into the airway milieu. However, it still needs to be investigated whether such a mechanism exists when NMDA-Rs are activated under chronic inflammatory conditions such as asthma.

Studies done by Perez-zoghbi *et al* and others in murine lung slices suggest changes in $[Ca^{2+}]_i$ mobilization underlying NO-dependent relaxation of bronchial airways [140]. In concurrence with our studies on HASM cells, we observed a significant induction in obligatory *nr1* subunit mRNA expression in murine airway slices, both in TNF-treated and HDM-exposed samples. This further supports our belief that NMDA-R expression is upregulated in mouse airways exposed to inflammatory cytokines, which might correspond to increased calcium flow into the cells. However, we still need to provide experimental

evidence in support of such assumptions. Contrary to our observations in HASM cells, relative expression of regulatory (*nr2a and nr2c*) subunits seems to be down-regulated under inflammatory conditions, along with inhibitory *nr3b* subunit. Also, we observed an increase in *nr3a* subunit. It is known that NMDA-R exhibit heterogeneous subunit expression and functional behavior depending on their localization in neuronal and/or non-neuronal tissues [185, 186]. For the first time, we demonstrate that NMDA-R subunit expression and receptor regulation exhibit considerable similarity in mouse airways and human ASM cells. However, presence of *nr2a* and absence of *nr2d* subunit expression in murine airways suggests existence of an interspecies difference in NMDA-R composition within lungs and airways. This seems to be in concurrence with observations in other multimeric receptor assemblies such as γ-aminobutyric acid (GABA) – A ion channels and human IgG (Fcγ) receptors [317, 318]. It is possible that some of our observations in mice, and in fact other animal models, may be not prove relevant in humans. Therefore, a cautionary approach needs to be undertaken to address discrepancies related to studying human NMDA-R function using animal models. One possible alternative is to design experiments using transgenic mice expressing humanized NMDA-Rs.

In conclusion, we demonstrate differential induction of multimeric NMDA-R ion channels in HASM cells specifically by TNF. This differential increase in NMDA-R expression was associated with increased $[Ca^{2+}]_i$ in these cells, which was also attributed to plausible TNF-dependent reorganization of regulatory and inhibitory NMDA-R subunits. Further, selective pharmacological activation of NMDA-Rs on smooth muscle cells potentiates Ca^{2+} - mediated bronchial relaxation *ex vivo* in murine airways. This

relaxing effect partially depends on mechanisms mediated through NOS and COX pathways.

CHAPTER 6.0

GENERAL CONCLUSIONS, SIGNIFICANCE AND FUTURE DIRECTIONS

6.1.0 GENERAL SUMMARY AND SIGNIFICANCE

Classically, dysregulated immune responses have been considered the basis for orchestrating and perpetuating AHR and bronchial obstruction observed in chronic airway disorders. In lungs and airways, inflammation is mediated primarily through infiltration and accumulation of immune cells including neutrophils, eosinophils, macrophages, dendritic cells, basophils, and mast cells. In addition, Th1 and Th2 lymphocytes are present in the airway lumen, and their presence and cytokine profile correlates with disease severity [13]. Immune cells collectively release an array of pro-inflammatory mediators and proteases that initiate cycles of “tissue injury and wound repair” in structural tissues surrounding the airways, a feature defined as AR.

In the recent years, susbstantial research done on airway contractility using sophisticated techniques and allergen-induced animal models of asthma compelled researchers to redefine the occurence of AHR in airways and the contribution of ASM tissue. ASM is critical for managing airway tone and regulates airway contraction and relaxation in response to spasmogens and relaxing agents that can circulate and found in the local milieu [319]. Emerging evidence suggests ASM cells are intrinsically abnormal or “hypercontractile” in certain asthmatics, which might be either innate or acquired due

to chronic exposure to inflammation and other factors such as viral infections[320]. Additionally, ASM once considered as a "passive bystander", has now emerged as a multifunctional tissue owing to its ability to demonstrate functional plasticity and an "inflammatory-cell like" phenotype [65]. Considering the fact that ASM hypertrophy and hyperplasia are noticeable clinical features of asthma, this tissue presents a potentially impactful therapeutic target.

In the ongoing efforts to discover extrinsic stimuli released by surrounding structural cells or nerve innervations that affect ASM phenotype, neuroeffector mechanisms have been identified as notable contributors to ASM function. These neuroeffector mechanisms are mediated through various neuromediators including excitatory neurotransmitters such as glutamate and their receptors. In our current study, we investigated the role of a family of neuronal glutamate receptor complexes known as NMDA-Rs in ASM cells *in vitro* and *ex vivo* methodologies. Prior observations by Said *et al* and Strapkova *et al* suggest an indirect association between NMDA-R and ASM cells, as NMDA-R stimulation can induce bronchial hyperresponsiveness in animal lungs [79, 256]. Correspondingly, identification of multiple NMDA-R subunits in different regions of rat lungs by Dickman *et al [244]* further supported the notion that a functional NMDA-R mediated glutamatergic system exists in airways. ***However, the presence of NMDA-R in human lungs, in particular HASM cells, has remained unconfirmed prior to our studies. A few questions that formed central crux to this thesis included (1) which subunits of NMDA-R are expressed in human ASM cells?; (2) are NMDA-R expressed on human ASM cells functional?; (3) what is the physiological role of NMDA-R in ASM***

cells?; (4) how are NMDA-Rs regulated in human ASM cells?; and (5) how does activation of NMDA-Rs affect AHR?

NMDA-R EXPRESSION IN HASM CELLS IS ASSOCIATED WITH AIRWAY CONTRACTION

NMDA-Rs are multi-subunit receptor assemblies permeable to cations Na^+, K^+ and Ca^{2+}. As described in chapter 4.0, we first profiled transcript expression of different receptor subtypes in HASM cells by qPCR. Based on our findings, we confirmed that HASM cells express almost all NMDA-R subunits (excluding NR2A and NR2B). The mRNA expression of NR2D and NR3B subunits is high, which indicates the presence of low calcium conductance channels with reduced sensitivity to external Mg^{2+} blockade may be formed [321]. Our data indicate that the transcript abundance of obligatory NR1 subunit is actually rather low in HASM cells, compared to SK-N-SH cells used as a positive control. Nonetheless, we were able to readily detect NR1 subunit protein in these cells using WB, IF and FACS. Previously, Nassar *et al* demonstrated positive NR1 protein expression in bronchial SMCs from human tracheal tissues. However, their staining indicates inconsistencies with respect to localization of NR1 protein specifically to SMC layer around airways [322]. For the first time, we showed that HASM cells are capable of expressing other NMDA-R subunits apart from NR1.

Formation of functional NMDA-Rs requires assembly of 2 NR1 subunits with either 2 regulatory NR2 subunits or 1 regulatory NR2 and 1 inhibitory NR3 subunits. In

addition, 2 NR1 subunits can combine with 2 NR3 subunits, thereby forming a unique glycine-responsive NMDA channels. However, we have limited information on the functional role or existence of such a receptor assembly. Agonist-binding to NMDA-Rs opens the ion pore and allows Ca^{2+} ion flow into the cell. For the first time, we showed that NMDA-R forms a novel ROCC in HASM cells, and receptor activation is essentially involved in the maintenance of intracellular Ca^{2+} homeostasis. NMDA receptors expressed on HASM cells demonstrated concentration-dependent Ca^{2+} mobilization when stimulated with increasing concentrations of specific pharmacological agonist NMDA. While these Ca^{2+} currents showed a delayed start, they were sustained over the entire time of measurement. While a similar pattern of Ca^{2+} flow was observed when TRPC3 channels were activated in HASM cells [323], additional investigations are warranted to determine whether such responses are a general feature of receptor-operated ion channels.

We have also shown for the first time that NMDA-R activation induces contraction of human SMCs, which was further confirmed using *ex vivo* murine TCLS. However, perhaps our most novel finding is the presence of intercellular heterogeneity in $[Ca^{2+}]_i$ responses in HASM cells when stimulated with NMDA. *In vivo*, ASM cells are believed to originate from heterogenous cell populations and exist as phenotypically diverse sub-populations with distinct functions [11, 258]. Based on our observations, we can speculate that NMDA-R subunit composition may be different in each of these ASM sub-populations leading to differential intracellular Ca^{2+} flow patterns. Another alternative explanation is

ASM cells, based on their phenotype, vary in terms of NMDA-R expression and membrane localization leading to varied Ca^{2+} responses. While both possibilities exist in neurons, we still need to confirm our speculations in HASM cells. Cell-cell heterogeneity is of interest as it suggests that NMDA-R may also reflect diverse phenotype and functional features of individual ASM cells. For example, secretory function versus proliferation versus contraction of cells within particular environmental circumstances. Future studies that determine whether unique Ca^{2+} responses are associated with distinct ASM cell functions are needed.

REGULATION OF NMDA-R EXPRESSION AND FUNCTION BY TNF

A critical role for proinflammatory cytokine TNF as the principal mediator of lung inflammation and AHR is well established [300]. In ASM cells, deleterious effects of TNF involve deregulating cellular Ca^{2+} homeostasis via induction of ROCCs such as TRPC3 and CD38 leading to increased Ca^{2+} flux [59, 323]. Considering that TNF potentiates NMDA-R-mediated glutamate excitotoxicity in cultured neurons [294], we proceeded to investigate the effect of TNF on the expression of NMDA-R subunits and their physiological function in HASM cells *(chapter 5.0)*.

For the first time, we provided evidence that NMDA-R subunit abundance in HASM cells is differentially modulated by TNF. In particular, NR1 protein expression increased substantially indirectly corresponding to increased receptor formation on the cells. These alterations in receptor subunit expression translated into increased intracellular

Ca^{2+} flow together with altered agonist-induced Ca^{2+} response patterns in different ASM sub-populations. Based on our observations on NMDA-R subunit abundance, we speculate that TNF exposure causes a shift in NMDA-R subunit composition and alters receptor distribution on the membrane in these different cell populations. As a result, we observed a higher percentage of cells responding to agonist stimulation.

Interestingly, none of the pro-inflammatory cytokines we tested, except TNF, affected NMDA-R subunit expression indicating specificity in response to TNF in regulating NMDA-R expression in airway myocytes. Binding of TNF to its cell surface receptors TNF-RI or TNF-RII leads to recruitment of various intracellular adapter proteins and induction of multiple signaling pathways. These pathways culminate with activation of transcription factors nuclear factor kappa – B (NF-κB), activator protein -1 (AP-1) and cAMP response element binding (CREB) protein. Promoter regions and gene loci near transcription start sites corresponding to NR subunits contain multiple regions that facilitate nuclear binding of transcription factors mentioned above and regulate their gene transcription [324]. In neuronal cells, NMDA-R subunit gene transcription is also regulated by pro-inflammatory cytokines IL-6 and IL-1β, albeit indepedent of TNF [325]. Our studies on NR1 transcript abundance in TNF and IL-1β-exposed airway myocytes suggests the occurence of a similar phenomenon, as positions my current findings as a platform for future investigation on how inflammation can regulate NR subunit gene expression.

For the first time, our studies showed a direct physiological relevance of NMDA-R activation in an allergen-induced mouse model of allergic inflammation. While available information is scarce on the role of glutamate NMDA ion channels in propagating AHR, Strapkova *et al* and Antosova *et al* tried to evaluate their functional role during an allergic airway response using an ovalbumin-induced airway inflammation model in guinea pigs [256, 303]. However, there exists some limitations to these studies. Firstly, intraperitoneal ovalbumin sensitization model does not necessary represent the mode of allergen exposure occuring in humans [301]. Secondly, NMDA was admininstered intraperitoneally to whole animal prior to ovalbumin exposure. Such a protocol doesnot represent direct impact of NMDA-R activation in inflamed lungs and airways, particularly in ASM cells. To address these concerns, we used intranasal HDM administration to induce acute allergic lung inflammation.

Owing to their ability to represent 3-dimensional histological organization existing in an intact lung, an *ex vivo* TCLS method was used to assess NMDA effect on airways. Our results demonstrated that NMDA-R activation induces bronchodilation in murine airways exposed to inflammatory mediators, and this effect is dependent on the presence of extracellular Ca^{2+}. Further, we demonstrated that iNOS and COX-2 participate in mediating bronchodilation in airway slices from TNF-treated and HDM-exposed mice. It is known that stimulation of NMDA-R causes vasodilation, both in cerebral and renal arteries [326, 327]. This response is coupled to NOS activation and NO release. A similar association between NMDA receptor-mediated glutamatergic system and nitrergic signaling in bronchial tissues was demonstrated in studies by Said *et al* and Antosova *et al*,

thereby corroborating our observations in mice [252, 303]. We showed that these responses are exclusively dependent on the presence of extracellular Ca^{2+} in the milieu. Unexpectedly, mice lung tissues expressed a completely different NMDA-R subunit composition from human ASM cells, which was significantly downregulated by inflammatory stimuli. It seems NMDA-R subunit regulatory machinery is slightly different in human and mouse lungs. These findings were interesting as they point to inconsistencies observed when using animal models to understand the physiology of NMDA-Rs, or other receptor assemblies.

To conclude, we have shown that functional NMDA-R are present on human ASM cells and that they act as novel ROCCs mediating intracellular Ca^{2+} entry. We further evaluated the factors responsible for regulation of NMDA-Rs in HASM cells and demonstrated an appreciable effect of TNF in modifying receptor expression and NMDA-induced Ca^{2+} response patterns. This knowledge equips us to further understand the nuances underlying regulated and deregulated Ca^{2+} entry mechanisms in ASM cells in normal and pathological circumstances. ASM cells are known, at least under *in vitro* culture conditions, to exhibit morphological and phenotypic plasticity and demonstrate cellular heterogeneity when responding to different contractile and relaxation agonists [328, 329]. Our observations suggest changes in agonist-induced cellular Ca^{2+} response patterns occuring partly due to differential receptor expression, configuration and localization as one possible mechanism explaining such intercellular heterogeneity. Similar studies on other ROCE mechanisms, if done in future, will provide us further insights into this phenomenon. Additionally, our studies point to interspecies discrepancy that exists

between humans and mouse pertaining to NMDA-R subunit expression, a phenomenon quite frequently observed in multimeric receptor assemblies.

6.2.0 LIMITATIONS OF OUR RESEARCH FINDINGS

Experimental observations summarized in this thesis underline the importance of glutamatergic signaling in lung health and disease. However, there exists certain limitations in our research findings that warrant an alternate explanation. For instance, we used *in vitro* cultured primary and immortalized human ASM cell lines to characterize the expression of different NMDA-R subunits and explore their functional relevance. It is essential to acknowledge the fact that some of our experimental outcomes might not exactly represent an *in vivo* physiological scenario.

Whole lung mechanics studies using the Flexivent system demonstrated no effect of NMDA stimulation both in control versus HDM-exposed mice. NMDA-R are Ca^{2+}-entry channels that mobilize small, yet sustained Ca^{2+} flow into the cells compared to conventional contractile agonists. Additionally, we believe NMDA-mediated responses in mouse airways are subtle and exhibit cellular heterogeneity across the tissue. As a result, it is possible that these responses are beyond the detectable threshold of the device. TCLS presents an ideal *ex vivo* alternative to study such diminutive agonist-induced small airway responsiveness at a microscopic level [128].

Currently, we have little information on the NMDA-R subunits expressed in normal and asthmatic human ASM tissues, considering subunit expression is speculated to vary within different regions of the lung. There is a need to improve our knowledge in this regard to further understand the role of NMDA-Rs in human lungs and airways.

Another important limitation is lack of information about the concentration of different NMDA-R agonists and co-agonists in chronic airway pathologies. As briefed in Chapter 1, several lines of indirect evidence suggests glutamate and kynurenine levels are elevated in bronchial asthma patients [206, 330]. At the same time, increased levels of excitatory neuromediators glutamate, glycine and kynurenines were observed in various models of lung injury [331, 332] and lung cancer [333, 334]. Contrastingly, decreased plasma levels of glutamate and glycine were observed in few studies involving children suffering from asthma [335]. While these observations suggest differential regulation of amino acid levels in bronchial pathologies, they necessarily neither correlate with disease occurence nor severity. Therefore, additional investigations are warranted using BALF, serum and lung exudate samples from normal controls and patients to address these discrepancies to further evaluate the contribution of neurotransmitters and their receptors in airway diseases.

6.3.0 FUTURE DIRECTIONS

In the past decade, there has been a renewed interest in field to understand the role of neurotransmitters, in particular glutamatergic signaling system via NMDA-Rs in the

peripheral organs[241]. In particular, their importance in modulating chronic diseases presents exciting and novel therapeutic targets [173]. Data presented in this thesis broadly outlines first ever systematic description of functionally-responsive NMDA-R in ASM cells using *in vitro* and *ex vivo* approaches.. Further, our study provides a novel role of NMDA-R in regulating murine airway responses. Ever since Said *et al* speculated the role of NMDA-R mediated excitotoxic effects of glutamate in lungs and their involvement in evoking AHR in bronchial tissues [79], limited advancements have been made to understand the expression, subunit assembly, regulation and functional importance of these receptors. Interstingly, all these studies used allergen-sensitized or agonist-treated *in vivo* animal models to examine NMDA-R role in asthma [96, 252, 256, 303]. The existence of interspecies variability in NMDA-R subunits, as demonstrated in our experimental observations, further complicates our understanding. In this regard, there are several key areas that need to be addressed as outlined below.

1. Although levels of glutamate and other NMDA-R agonists are elevated in bronchial asthmatics, no report exists on receptor expression in normal, asthmatic and COPD lungs. Studies done by Dickman *et al* have demonstrated a differential receptor subunit composition in proximal, central and distal parts of a rat lung [244]. Based on the findings from neuronal tissues, we believe a similar level of complexity exists in human lungs. To address these concerns, it is important to profile NMDA-Rs in control and diseased human lungs, particularly in ASM tissue, in a large population study to correlate their relevance to disease.

2. Inside the CNS and other non-neuronal tissues, NMDA-Rs are suggested to be expressed by various cell types including epithelial cells, and fibroblasts [336, 337]. We need to describe the expression and function of NMDA-Rs in these different cell types inside lungs to appreciate their contribution in AHR, airway inflammation and remodeling.

3. Elucidate NMDA-R induced intracellular signaling networks and gene expression profiles in ASM cells that mediate airway contractility or bronchodilation when stimulated with specific pharmacological agonist.

4. ASM cells exhibit phenotypic plasticity and switch between contractile, proliferative and synthetic. Observations from our studies confirm NMDA-R's role in airway contractility. However, studies from other cell types including neurons indicate NMDA-R activation stimulates cellular proliferation and secretion of multiple pro-inflammatory mediators [338-341]. We need to evaluate whether NMDA-Rs are involved in mediating ASM proliferative and synthetic phenotypes.

7.0 CHAPTER 7

APPENDIX

A1 NMDA-R subunit expression in SK-N-SH Cells

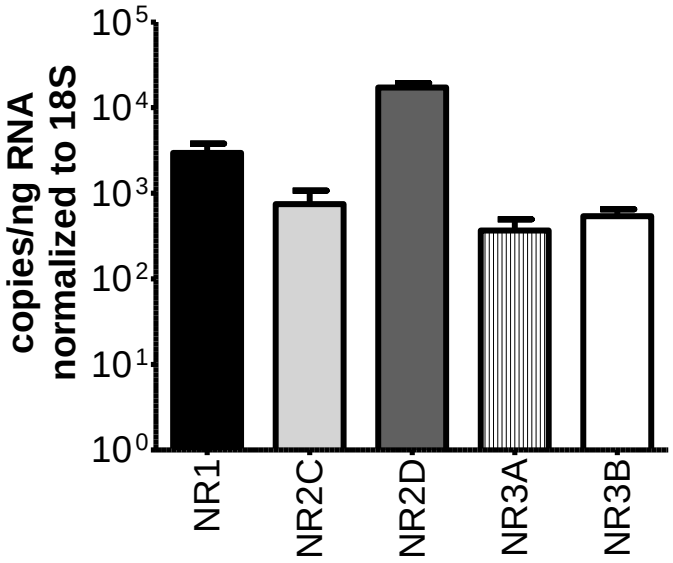


Figure A1: *NMDA-R subunit expression in SK-N-SH cells* – qPCR was performed for mRNA isolated from cultured SK-N-SH cells using subunit specific primers. Abundance of mRNA for respective NMDA-R subunits was expressed as copies/ng total RNA normalized to 18S.

A2 IFNγ and IL-4 do not regulate NMDA-R subunit expression in HASM Cells

We investigated whether NMDA-R subunit expression is regulated by IFNγ and IL-4 considered as classical Th1 and Th2 cytokines respectively. For this, RNA samples of cultured HASM cells were obtained following 48h stimulation with IFNγ and IL-4

individually @ 10ng/mL. Upon performing qPCR, we observed that both IFNγ and IL-4 had no effect in altering expression of any NMDA-R subunit (fig A2; A-E, n=6), when compared with unstimulated medium controls. Additionally, we evaluated the effect of other proinflammatory cytokines IL-1β and TGF-β in regulating mRNA expression of NMDA-R subunits.10ng/mL IL-1β exposure induced NR1 mRNA levels in HASM cells (fig A2; F, n=3). thereby demonstrating an expression pattern similar to TNF treatment. Also, we did not observe any significant augmentation in subunit expression when cells were treated with a combination of IL-1β and TNF, suggesting IL-1β acts independently of TNF in regulating NMDA-R subunit expression. NR3B mRNA expression did not alter with IL-1β treatment (fig A2; G, n=3). 2.5ng/mL TGF-β exposure had no effect on NR1 and NR3B abundance (fig A2; H-I, n=3). Taken together, we demonstrate that TNF, which is considered as a classical prototype cytokine representing inflammation, selectively regulates transcript abundance of multiple NMDA-R subunits.

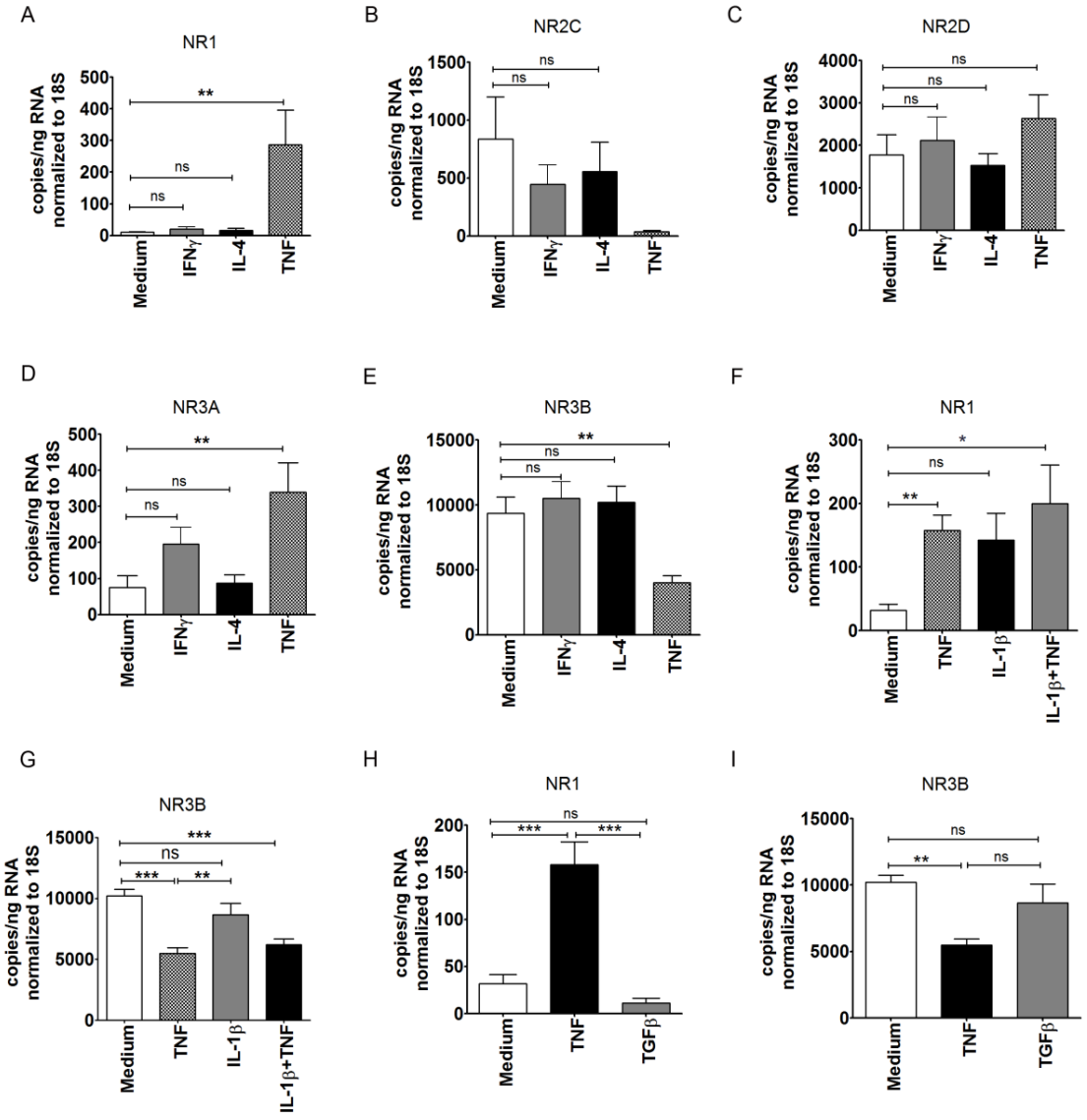


Figure A2: *Effect of pro-inflammatory cytokines on NMDA-R subunit mRNA expression in airway myocytes* - qPCR was performed using subunit specific primers in mRNA isolated from cultured airway myocytes treated for 48h with IFN-γ (10ng/mL), IL-4 (10ng/mL), TNF (10ng/mL), IL-10 (10ng/mL) and TGF-β (2.5ng/mL). Data represent mean ± SEM of 3-6 different experiments performed in triplicates under same experimental conditions. Data was analysed by One-way ANOVA with Bonferroni's multiple comparison tests (***P<0.001; **P<0.01; *P<0.05; ns=non significant). Histograms indicate IFN-γ, IL-4 (A-E, n=6) and TGF-β (H-I, n=3) exposure did not change transcript expression of NMDA-R subunits compared to untreated medium controls. Exposure to IL-1β induced NR1 expression (F) but did not alter NR3B levels (G).

A3 NMDA-R activation does not induce airway contractility in murine tracheal segments

Endogenously, NO is generated during the breakdown of aminoacid L-Arginine, which is an nicotinamide adenine dinucleotide phosphate (NADPH) -dependent reaction catalyzed by NOS [306]. One of the important contributors of NO inside airway microenvironment is the epithelial layer surrounding the ASM [306, 312]. Based on our observations that inhibition of NOS blocks NMDA-R-mediated airway dilation, we wanted to investigate the contribution of airway epithelium to these responses. For this, we monitored contractile responses in naive mice to cumulative dose responses of NMDA (15.6µM – 3mM), both in epithelium-containing and epithelium-denuded murine tracheal segments using myograph studies (Fig A3; A). Interestingly, neither of the tracheal segments demonstrated any bronchial contractility with NMDA stimulation. As a positive control, we used % mean maximal isometric force demonstrated with 1µM MCh (~138% of force generated by KCl). To confirm the presence of NMDA-R in tracheal rings, we determined NR1 abundance by RT-PCR. Both epithelium-containing and denuded samples demonstrated NR1 mRNA abundance (Fig A3; B). However, the relative abundance of NR1 transcript was similar in both samples, suggesting that NR1 presence is primarily localized in ASM tissue. Further, when 18h TNF-treated trachea were mounted onto the transducers and stimulated with NMDA, we observed a slight reduction in maximal force compared to matched control tracheal segments suggesting airway relaxation (Fig A3; C). However, the numbers were considerably insignificant in comparison to MCh. Similarly, there was no significant change observed in epithelium-denuded rings. In summary, we

were unable to conclude whether NMDA-R activation affects airway responses in mouse tracheal segments. Further, we were unable to determine role of epithelial layer in regulating bronchial dilation in murine lung slices. However, we still cannot negate the role of NMDA-R in regulating airway responses.

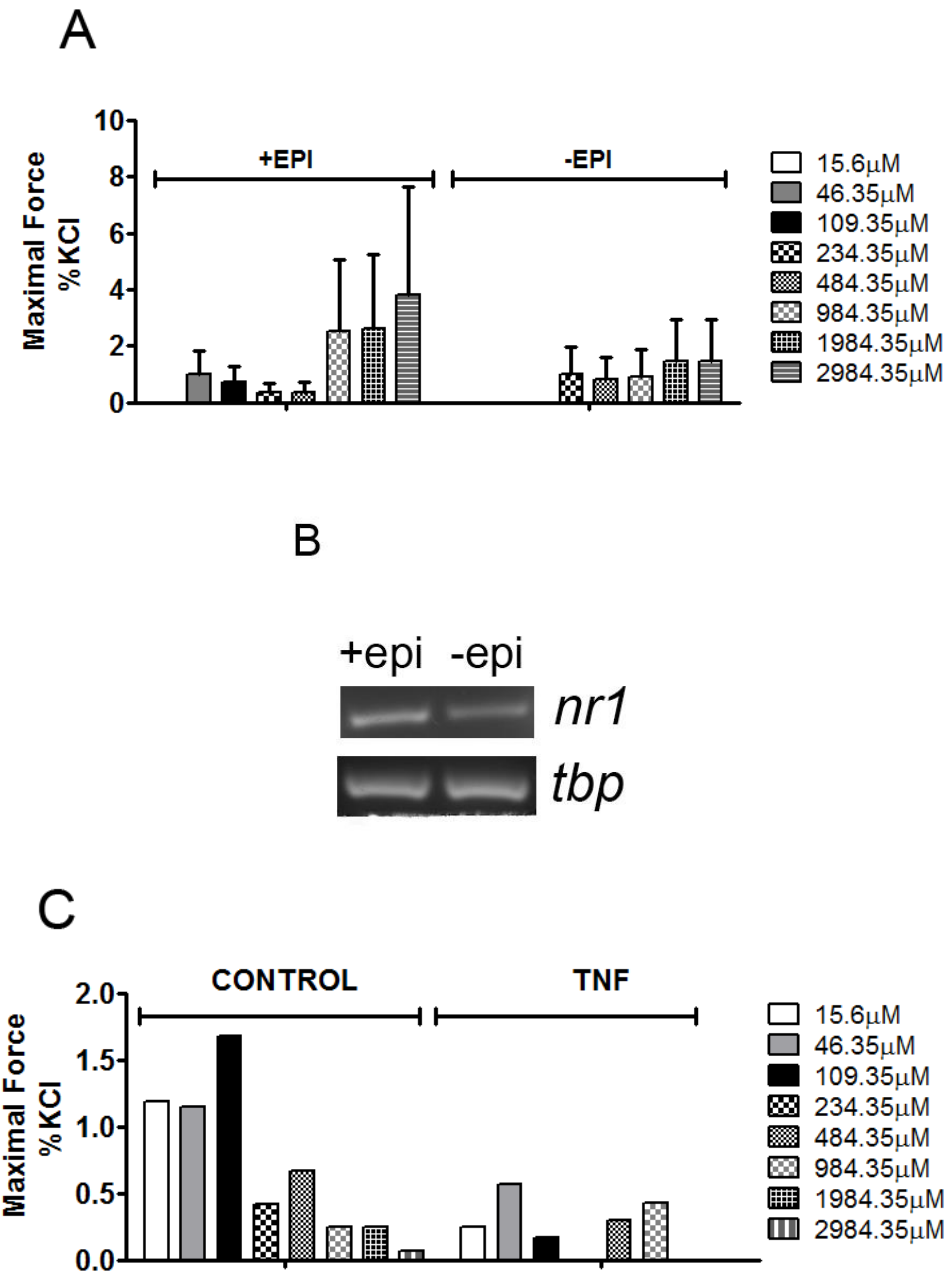


Figure A3: *NMDA-R activation does not induce airway contractility in murine tracheal segments* – Tracheal rings isolated from 8-10wk old Balb/c mice were mounted onto a organ transducer system and equilibrated for 90-120min, with intermittent intillations of K-H buffer containing 63mM KCl (at ~20min interval for 3 times).Resting tension was optimized at 0.6mN. NMDA concentration response studies were done using epithelium-containing (+ epi) and epithelium-denuded (- epi) rings. (A) NMDA-concentration response studies did not elicit any airway contraction both in + epi and – epi rings (rings obtained from 6 mice for these studies). (B) RT-PCR data shows equal *nr1* abundance in + epi and – epi samples. We used *tbp* as a reference control gene. (C) Epithelium-containing tracheal rings were isolated and cultured in DMEM (without FBS) for 18h with or without 10ng/mL TNF. Myograph studies show no change in responsiveness with TNF, when stimulated with increasing dose of NMDA (Rings from 2 mice were used). Data represent mean $\pm$ SEM.

Table A4: Summary of primer sequences used in qPCR – Primers were designed using Primer Quest (IDT Technologies Inc) and verified by Primer Blast (NCBI). Specific amplification was confirmed by PCR and one-dimensional agarose gel electrophoresis.

Gene	Species	Primer Pairs (5'----3')	Amplicon Size (bp)	Anneal (°C)
18S	Human	AAAGGAATTGACGGAAGGGCACCA ACCAGACAAATCGCTCCACCAACT	174	60
GRIN1	Human	AATGGCACCCACGTCATCCCTAAT AACTCCTCCTTGCATGTCCCATCA	170	60
GRIN2A	Human	TTGCCACCACCGGTTATGGAATTG AATACGCCCGCCATGTTGTCAATG	196	60
GRIN2B	Human	GGCTCCAGCAATGGGCATGTTTAT AGCAAATGGGAACCAAGTTCACCC	162	60
GRIN2C	Human	TAACTACCGTGACATGCACACCCA TTGCCTGCCATGTAGTTGAGGACA	135	60
GRIN2D	Human	TCAATAATTCGGTGCCCGTGGAGA TGTGTAGCTGGCGAGGAAGATGA	101	60
GRIN3A	Human	ACGGAGACTTTGCAAATGGGCATC ATTCGTGGTAGCAGCAGCCTGTAT	128	60
GRIN3B	Human	TTCATCATGGACAAGTCGCTCCTG AGGACTTGTAGCGGCTGATGAACT	166	60
GRIN1	Mouse	CCAGATGTCCACCAGACTAAAG CCGTACAGATCACCTTCTTGAC	137	60
GRIN2A	Mouse	GCGCAGAACGCGGCG AGCCTCTTGGTCCGTATCATCT	261	64
GRIN2B	Mouse	ACATGCGCTCTCCCTTAATC AGAGATGATGGAAGTCATCTTTC	259	60

GRIN2C	Mouse	CATTGGGTCTGGCAAAGTCT CACCTCGTTCTTCTCGTTATGG	258	60
GRIN2D	Mouse	GAGTACGACTGGACATCCTTTG CCACCATGAACCAGACGTAG	286	60
GRIN3A	Mouse	ATGCCAGGAAGACTGGAATATC ATGAAGATCAGGAGGTGATAGC	253	60
GRIN3B	Mouse	GTTTCTGAGCAACACCTCATTTC GAGTTACCACTCGCAGCTTT	245	60
COX-1	Mouse	CCAGTCAATCCCTGTTGTTACT GATCTCAGGGATGGTACAGTTG	129	60
COX-2	Mouse	CCTTCTCCAACCTCTCCTACTA GGAAGCTCCTTATTTCCCTTCA	94	60
iNOS	Mouse	CTTGGTGAAAGTGGTGTTCTTTG TCAGACTTCCCTGTCTCAGTAG	102	60

8.0 CHAPTER 8

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