Genetic Regulation of Endoplasmic Reticulum Chaperones and Pro-Inflammatory cytokines by neuronal α4β2 nicotinic receptors

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Abstract

α4β2 Nicotinic acetylcholine receptors play important roles in the reward pathways for nicotine. We investigated whether receptor up-regulation of α4β2 receptors involves expression changes for non-receptor genes. In a microarray analysis, 10µM nicotine altered expression of 41 genes at 0.25, 1, 8 and 24h in hα4β2 SHEP1 cells. Half of the genes were related to endoplasmic reticulum (ER) chaperones and the remaining genes belonged to inflammation and immune-response pathways.

The first objective was screening genes that alter surface α4β2 expression using correlation analysis and RNA interference. Antagonists potentiate nicotine-induced α4β2 upregulation, and correlation analysis showed that antagonists alone or in combination with nicotine suppressed an ER chaperone CRELD2 message while increasing surface expressing α4β2 receptors. siRNA knockdown of CRELD2 increased basal α4β2 receptor expression, and antagonists alone decreased CRELD2 mRNA in wild type SHEP1 cells lacking α4β2 receptors. These data suggest that ER proteins such as CRELD2 decreases surface α4β2 expression, and may explain antagonist actions in nicotine-induced receptor up-regulation.

The second objective was investigating the signaling pathways downstream of α4β2 receptors leading to suppression of immune responses. Nicotine suppresses inflammatory cytokines and chemokines in hα4β2 SHEP1 cells but not in wild type SHEP1 cells. Quantitative RT-PCR (qPCR) corroborated nicotinic suppression of pro-inflammatory cytokines (PICs) IL-1β and IL-6. 10 µM nicotine suppressed basal IL-1β and IL-6 protein expression by blocking NFκB translocation. Nicotine dose-dependently attenuated lipopolysaccharide (LPS)-induced NFκB translocation, IκBα phosphorylation and PIC production. A cell-permeable calcium chelator BAPTA-AM, adenylate cyclase stimulant forskolin and a specific PKA inhibitor PKI 14-22 AMIDE failed to block the effects of nicotine on LPS-induced NFκB translocation and IκBα phosphorylation. The specific JAK2 inhibitor AG-490 and STAT3 inhibitor NSC74859 significantly blocked the anti-inflammatory effects of nicotine. These findings reveal a calcium-and cAMP-PKA independent signaling cascade and suggest a role for JAK2-STAT3 transduction in α4β2-mediated anti-inflammatory actions against endotoxin-induced inflammation.
Nicotine exposure decreased PIC production while upregulating α4β2 receptors. This negative association between nicotine-induced increases in α4β2 receptors and immune suppression may explain the neuroprotective effects observed in chronic smokers against neurodegenerative diseases such as Alzheimer’s and Parkinson’s disease.
# Table of contents

Abbreviations used ................................................................................................................................................ vii
List of tables ............................................................................................................................................................ x
List of figures .......................................................................................................................................................... x
Acknowledgements ............................................................................................................................................... xii
Preface .................................................................................................................................................................. xiii

<table>
<thead>
<tr>
<th>Chapter 1</th>
<th>.................................................................................................................................................................</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Problem statement ........................................................................................................................................</td>
<td>1</td>
</tr>
<tr>
<td>2.</td>
<td>Background and Significance .......................................................................................................................</td>
<td>2</td>
</tr>
<tr>
<td>2.1</td>
<td>Nicotinic receptors. ..................................................................................................................................</td>
<td>2</td>
</tr>
<tr>
<td>2.2</td>
<td>High affinity binding ................................................................................................................................</td>
<td>3</td>
</tr>
<tr>
<td>2.3</td>
<td>Composition and stoichiometry of αβ2 receptors .......................................................................................</td>
<td>5</td>
</tr>
<tr>
<td>2.4</td>
<td>αβ2 upregulation .....................................................................................................................................</td>
<td>6</td>
</tr>
<tr>
<td>2.5</td>
<td>Proposed mechanisms for αβ2 upregulation ...............................................................................................</td>
<td>11</td>
</tr>
<tr>
<td>3.</td>
<td>αβ2 Upregulation and Nicotine addiction ..................................................................................................</td>
<td>20</td>
</tr>
<tr>
<td>4.</td>
<td>Objectives and specific aims .....................................................................................................................</td>
<td>22</td>
</tr>
<tr>
<td>5.</td>
<td>Materials and methods ...............................................................................................................................</td>
<td>25</td>
</tr>
<tr>
<td>6.</td>
<td>Results .......................................................................................................................................................</td>
<td>27</td>
</tr>
<tr>
<td>6.1</td>
<td>Nicotine-induced upregulation of αβ2nAChRs ...........................................................................................</td>
<td>27</td>
</tr>
<tr>
<td>6.2</td>
<td>Microarray analysis of nicotine-induced αβ2 upregulation ....................................................................</td>
<td>28</td>
</tr>
<tr>
<td>6.3</td>
<td>Validation of transcript changes using qPCR ...........................................................................................</td>
<td>29</td>
</tr>
<tr>
<td>6.4</td>
<td>Antagonists potentiate nicotine-induced upregulation ............................................................................</td>
<td>31</td>
</tr>
<tr>
<td>6.5</td>
<td>Influence of CRELD2 on αβ2 upregulation ...............................................................................................</td>
<td>34</td>
</tr>
<tr>
<td>7.</td>
<td>Discussion ..................................................................................................................................................</td>
<td>35</td>
</tr>
<tr>
<td>8.</td>
<td>Future directions .......................................................................................................................................</td>
<td>39</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 2</th>
<th>.................................................................................................................................................................</th>
<th>41</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.</td>
<td>Problem statement ........................................................................................................................................</td>
<td>41</td>
</tr>
<tr>
<td>10.</td>
<td>Glossary ...................................................................................................................................................</td>
<td>42</td>
</tr>
<tr>
<td>11.</td>
<td>Background and significance .....................................................................................................................</td>
<td>45</td>
</tr>
<tr>
<td>11.1</td>
<td>CNS is ‘immune-privileged’ ......................................................................................................................</td>
<td>45</td>
</tr>
<tr>
<td>11.2</td>
<td>‘Off’ signal is as essential as ‘On’ signal ..................................................................................................</td>
<td>46</td>
</tr>
<tr>
<td>11.3</td>
<td>Signaling pathways ...................................................................................................................................</td>
<td>47</td>
</tr>
<tr>
<td>11.4</td>
<td>Inflammatory reflex ..................................................................................................................................</td>
<td>53</td>
</tr>
<tr>
<td>11.5</td>
<td>Nicotine is immunosuppressive and anti-inflammatory ...........................................................................</td>
<td>55</td>
</tr>
</tbody>
</table>
12. Objectives and specific aims.......................................................................................................................... 57
13. Material and methods....................................................................................................................................... 60
14. Results............................................................................................................................................................ 62
  14.1. Nicotine suppresses cytokine production ............................................................................................... 62
  14.2. Antagonists block nicotine-induced suppression of cytokines ............................................................. 64
  14.3. Nicotine inhibits basal NFκB transactivation ......................................................................................... 65
  14.4. Nicotine blocks LPS-induced NFκB pathway ......................................................................................... 67
  14.5. Ca2+ influx does not influence nicotine-induced cytokine suppression .................................................. 69
  14.6. cAMP-dependent PKA signaling .......................................................................................................... 73
  14.7. JAK2 mediates α4β2-mediated anti-inflammation .................................................................................. 74
  14.8. NSC74859 restores LPS-induced NFκB activation ................................................................................. 74
15. Discussion ...................................................................................................................................................... 77
16. Future Directions ........................................................................................................................................... 87
Conclusion .......................................................................................................................................................... 91
Bibliography ........................................................................................................................................................ 92
Abbreviations used

- $\alpha$-Bgt alpha-bungarotoxin
- AD Alzheimer’s disease
- ADHD attention-deficit hyperactivity disorder
- AFC antibody forming cells
- BBB blood brain barrier
- BiP immunoglobulin binding protein
- cAMP cyclic AMP
- CNS central nervous system
- COPD chronic obstructive pulmonary disease
- CRELD2 cysteine rich with EGF like domain2
- CREM cAMP response element modulator
- CXCL chemokine ligand
- CSF cerebrospinal fluid
- DA dopamine
- dH$\beta$E dihydro-beta-erythroidine
- d-TC tubocurarine/curare
- ELISA enzyme linked immunosorbent assay
- ER endoplasmic reticulum
- ERAD endoplasmic reticulum associated degradation
- EAE experimental autoimmune encephalomyelitis
- GABA $\gamma$-Aminobutyric acid
- HD Huntington’s disease
- HERPUD1 Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1.
- HMGB1 high mobility group box 1
- Iк$\beta$α nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
• IKK  IκB kinase
• IL-1β  Interleukin 1 beta
• IL-6  Interleukin 6
• JAK  janus kinase
• KO  knock-out
• LDTg  laterodorsal tegmental nucleus
• LPS  lipopolysaccharide
• MAPK  mitogen-activated protein kinase
• Mec  mecamylamine.
• MHC  major histocompatibility complex
• MLA  Methyllycaconitine
• mRNA  messenger RNA
• MS  multiple sclerosis
• NAcc  nucleus accumbens
• nAChRs  nicotinic acetylcholine receptors
• NFκB  nuclear factor kappa B
• Nic  nicotine
• NMDAR  N-methyl-D-aspartic acid receptor
• PBS  phosphate buffered saline
• PD  Parkinson’s disease
• PDAI6  protein disulfide isomerase family A, member 6
• PKA  protein kinase A
• PKC  protein kinase C
• qPCR  quantitative real-time polymerase chain reaction
• RNAi  RNA interference
• SOCS  suppressors of cytokine synthesis
• SOD2  super oxide dismutase 2
• siRNA small interfering RNA
• SRB sulforhodamine B assay
• STAT Signal Transducers and Activator of Transcription
• TLR4 Toll-like receptor 4
• TNFα Tumor necrosis factor alpha
• UPR unfolded protein response
• VTA ventral tegmental area
List of tables

Table 1. Possible endoplasmic reticulum chaperones and miscellaneous genes ................................................................. 28
Table 2. Gene transcripts selected for validation by qPCR. ................................................................................................. 29
Table 3. qPCR primers .......................................................................................................................................................... 30
Table 4. Effect of antagonists dHβE and Mec on nicotine-induced gene alterations ........................................................... 33
Table 5. Accumulation of immune cells in the CNS contributes to inflammation-triggered neurodegeneration .......... 47
Table 6. Summarized results of nAChR-mediated suppression of cytokine production by cholinergic agonists ............... 56
Table 7. Inflammation related gene transcripts selected for validation by qPCR ................................................................. 63
Table 8. qPCR primers .......................................................................................................................................................... 63
Table 9. Inflammation and immune response genes ............................................................................................................. 64
Table 10. Effect of antagonists dHβE and Mec on nicotine-induced gene alterations. ........................................................ 65

List of figures

Figure 1. Pathway Assist™ visualization of all changed transcripts from the nicotine time-course experiments. ........xiv
Figure 2. Is α4β2 up-regulation an epiphenomenon or cellular adaptation? ........................................................................... 2
Figure 3. The structure of nAChRs ........................................................................................................................................ 3
Figure 4. Schematic of the cation π interaction ...................................................................................................................... 4
Figure 5. Schematic of four loops that define the aromatic binding pocket of nAChRs ........................................................ 4
Figure 6. Alignment for residues in four loops- ...................................................................................................................... 5
Figure 7. Benzene (6 π-electrons) .......................................................................................................................................... 3
Figure 8. 18F-2F-A85380 distribution volumes (DV) in the human brain ............................................................................ 7
Figure 9. Autoradiographic images of 125I-epibatidine binding after chronic nicotine exposure in rat pups ..................... 9
Figure 10. Expression of nAChRs determined by confocal microscopy. ............................................................................. 10
Figure 11. Effect of PICs IL-1β and TNFα exposure on α4, β2 and β4 nAChR assembly .......................................................... 11
Figure 12. Nicotine as a chaperone ....................................................................................................................................... 12
Figure 13. Nicotine-Receptor subunit interaction .................................................................................................................. 13
Figure 14. Agents that elevate intracellular cAMP induce upregulation ............................................................................. 15
Figure 15. Domain structure of Ubiquilin-1 ......................................................................................................................... 16
Figure 16. Schematic of CRELD2 domains and aminoacid sequence ................................................................................. 17
Figure 17. HERP .................................................................................................................................................................... 18
Figure 18. HERP has both ERSE and ATF-C/EBP sites. ......................................................................................................... 18
Figure 19. RIC3 protein domains .......................................................................................................................................... 19
Figure 20. Schematic of nicotine addiction and how α4β2 upregulation could influence nicotine-induced addiction. ....... 21
Figure 21. α4β2 upregulation in hα4β2 SHEP1 cells .............................................................................................................. 27
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Preface

Tobacco is one of the most widely abused drugs in the United States. Chronic smoking induces addiction, sensitization and tolerance, and smokers are at higher risk for lung cancer (Tsurutani et al., 2005; Mucha et al., 2006), coronary heart disease (Zhang et al., 2001) and COPD (Barnes et al., 2003). In contrast, cigarette smokers are less susceptible to the development of Parkinson’s disease (Fratiglioni and Wang, 2000) or Alzheimer’s disease (Wang et al., 1999). And, Schizophrenics self-medicate with nicotine to improve cognition and mental alertness (Ripoll et al., 2004). The table below lists the positive (+) and adverse effects (-) of chronic nicotine use.

Table 1. Tobacco smoking is associated with both beneficial and detrimental effects. (Dr. John Daly, NIH).

<table>
<thead>
<tr>
<th>Beneficial effects</th>
<th>Detrimental effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cognitive enhancement</td>
<td>Addiction, tolerance</td>
</tr>
<tr>
<td>Anxiolysis</td>
<td>GI distress</td>
</tr>
<tr>
<td>Neuroprotection</td>
<td>Hypothermia</td>
</tr>
<tr>
<td>Analgesia</td>
<td>Coronary diseases</td>
</tr>
<tr>
<td>Anti-psychotic</td>
<td>Respiratory diseases</td>
</tr>
<tr>
<td>Cerebro vasodilation</td>
<td>Seizures, Emesis</td>
</tr>
</tbody>
</table>

Nicotine, the major addictive component of tobacco smoke, interacts with high-affinity α4β2 nicotinic acetylcholine receptors (nAChRs) in the central nervous system (CNS). The pharmacology of nicotinic interaction with α4β2 is complicated because continuous exposure to low concentrations of nicotine increases the number of surface expressing receptors, a phenomenon known as upregulation. Pioneering work by Henry Lester’s laboratory at California Institute of Technology suggests that α4β2 upregulation might play a role in nicotine-induced addiction and tolerance, and neuroprotection (Nashmi et al., 2007; Xiao et al., 2009).

α4β2 upregulation is intriguing because both agonists and antagonists increase the surface receptor expression, and moreover, this is a post-transcriptional event. Although there is no change in the messages encoded for α4 and β2 subunits, there may be non-receptor gene changes in the endoplasmic reticulum (ER), cytoplasm and ubiquitin proteasomal system (UPS) that can provide clues on how upregulation occurs. Former students of our laboratory, Scott Leppanen and Adham Abutaha, utilized a microarray-based approach to identify nicotine-induced gene changes to
understand mechanisms underlying α4β2 upregulation. They investigated the effects of 10µM nicotine on gene expression in SHEP1 cells, stably transfected with human α4 and β2 subunits (hα4β2 SHEP1 cells) and found that nicotine exposure altered gene expression of 42 genes at 1, 8 or 24 h, with 8h showing the maximum altered gene expression (31/42 genes) when α4β2 receptors are starting to show significant up-regulation. Bioinformatics analysis categorized these genes into two clusters (Fig.1): 1) possible chaperones, transcription factors and probes with no known association with each other and 2) inflammation and immune response probes.

The following were the two objectives of my project:

i) To establish an in vitro screening paradigm to identify and test the function of genes altering surface α4β2-receptor expression from group 1 (Chapter 1).

ii) To investigate the signaling pathways downstream of α4β2 activation leading to suppression of IL-1β and IL-6, as examples from group 2, in hα4β2 SHEP1 cells (Chapter 2).
Chapter 1

1. Problem statement

Tobacco is one of the most widely abused drugs in the world. Nicotine is the positive reinforcing component of tobacco smoke and its addiction causes ~5 million deaths every year (Benowitz, 2009; Penton and Lester, 2009). The α4β2 nicotinic acetylcholine receptors (nAChRs) constitute the major high-affinity binding sites for nicotine in the central nervous system (CNS) (Dwoskin and Crooks, 2001; Walsh et al., 2008; Penton and Lester, 2009). The concentration of nicotine achieved in smokers’ brain (100-300nM) readily activates and desensitizes α4β2 receptors (Picciotto and Zoli, 2008; Riveles et al., 2008). Similar to the endogenous ligand acetylcholine, nicotine modulates the release of other neurotransmitters including dopamine, norepinephrine, serotonin, and glutamate (Mansvelder et al., 2003; Schilström et al., 2003; Penton and Lester, 2009; Quik et al., 2009).

Chronic nicotine induces up-regulation of α4β2 receptors in several brain regions including cortex, hippocampus, midbrain and striatum but not thalamus (Rowell and Li, 1997). Unlike normal agonist-induced receptor desensitization and down-regulation, the pharmacology of α4β2 up-regulation is complicated because chronic nicotine exposure increases $B_{\text{max}}$ without dramatically changing the affinity of the receptors (Wonnacott, 1990; Whiteaker et al., 1997). Further, both competitive and non-competitive antagonists either potentiate agonist-induced up-regulation or elicit the phenomenon themselves, limiting research tools to test upregulation (Gopalakrishnan et al., 1997; Buisson and Bertrand, 2001).

Epidemiological and post-mortem studies report appreciable increases in high affinity binding sites for nicotine in chronic smokers (Fratiglioni and Wang, 2000; Quik, 2004; Wullner et al., 2008). Although molecular events are unclear, researchers have established that up-regulation- 1) is a post-transcriptional event (Marks et al., 1992), 2) does not require channel activity (Gopalakrishnan et al., 1996). 3) is elicited by both permeable and impermeable agonists (Gopalakrishnan et al., 1997), 4) is potentiated but not blocked by antagonists, 5) is elicited by second messengers PKA and PKC (Gopalakrishnan et al., 1997), and 5) requires exocytic trafficking of receptors (Darsow et al., 2005) (facilitated by ubiquitin proteasome system, endoplasmic reticulum chaperones, cytosolic proteins etc).
Treatment strategies for smoking cessation are not effective. 80-90% of nicotine replacement therapy users relapse to smoking within 6 months (Rose, 1996). To develop better treatment strategies it is essential to understand the mechanisms underlying $\alpha_4\beta_2$ receptor upregulation. Despite significant progress made over the past decade, the phenomenon of $\alpha_4\beta_2$ up-regulation has been a hindrance and controlling the mechanisms underlying upregulation might lead to better smoking cessation strategies (Fig.2). Our proposed work involves identifying and investigating the function of genes involved in regulating the assembly, maturation and trafficking of $\alpha_4\beta_2$ nAChRs, which in turn will help to delineate mechanisms underlying $\alpha_4\beta_2$ upregulation. Because upregulation modulates nicotine-induced addiction, tolerance and sensitization (Tapper et al., 2004; Nashmi et al., 2007), future therapeutics for smoking cessation could aim to exploit or abate $\alpha_4\beta_2$ upregulation as an effective treatment strategy.

**2. Background and Significance**

2.1. Nicotinic receptors. These are hetero- or homo-pentamers that belong to a family of ligand-gated ion channels. A functional receptor is formed from assembly of five similar subunits for $\alpha_7$ or two $\alpha_4$ and three $\beta_2$ (high-affinity) or three $\alpha_4$ and two $\beta_2$ (low-affinity) for $\alpha_4\beta_2$ nAChRs (Zwart and Vijverberg, 1998). Acetylcholine is an endogenous ligand for nAChRs. The binding site is at the interface between two alpha subunits in case of $\alpha_7$ receptors and between alpha4 and beta2 subunits for $\alpha_4\beta_2$ receptors (see Fig.3).
The α4β2 is the predominant subtype followed by α7 in the CNS. Although nicotine from cigarette smoke enhances the risk for coronary diseases, lung cancer, arthritis and COPD, chronic smokers are at lower risk for ulcerative colitis, endometriosis, sarcoidosis, and neurodegenerative diseases (Sopori, 2002). Nicotine at doses achieved in a smoker’s brain activates, desensitizes and upregulates high-affinity binding α4β2 receptors (Wullner et al., 2008). α4β2 nAChRs are present on non-neuronal cells such as macrophages, microglia, lymphoid tissue and skin (Clementi et al., 2000; de Jonge and Ulloa, 2007).

**Figure 3. The structure of nAChRs.**

Nicotinic receptors belong to a large family of ligand-gated ion channels. They have an extracellular N-terminal domain, four transmembrane domains and a large cytoplasmic loop in between transmembranes 3 and 4. They form homo (α7)- or hetero (α4β2)- dimers and α-Bgt toxin binding differentiates α7 receptors from α4β2. α4β2 are high-affinity binding sites for acetylcholine and nicotine in the CNS. The ligand binding domain is at the interface between two α7 subunits for α7 receptors whereas between α4 and β2 for α4β2 receptors (Ancey et al., 2003; de Jonge and Ulloa, 2007).

2.2. High affinity binding. The endogenous ligand acetylcholine and nicotine show differential affinity to α4β2, and α7 and muscle type receptors (Schwartz and Kellar, 1983).

**Figure 4. Benzene (6 π-electrons)**
The differential affinity is contributed by strong cation-π interactions, which are weak or absent in α7 and muscle type receptors. Pioneering work conducted by Dougherty’s group to assess the high-affinity binding suggests that a combination of aromatic-amino (a type of cation-π interaction) and hydrogen bonding enhances the sensitivity of α4β2 receptors to nicotine (Xiu et al., 2009). A conventional cation-π interaction involves a non-covalent bond between aromatic ring systems π electrons and cations (Figures 4 &5). Experimental evidence supports the involvement of other simple-π systems (acetylene and ethylene) besides aromatic ring systems (Xiu et al., 2009).

![Figure 5. Schematic of the cation π interaction](image)

*(Cations including Na⁺, K⁺, Li⁺, Al³⁺, NH⁴⁺ etc)*

![Figure 6. Schematic of four loops that define the aromatic binding pocket of nAChRs](image)

Figure 6. Schematic of four loops that define the aromatic binding pocket of nAChRs (Xiu et al., 2009).
As shown in Figure 7, the highly conserved residues TyrA (tyrosine in Loop A), TryB, TyrC1, TyrC2 and TypD form the aromatic binding pocket. This aromatic pocket system of nAChRs provides a strong support for cation-\(\pi\) interactions. A classic example is the binding of \(\text{NH}_4^+\) group of nicotine with the aromatic \(\pi\)-system of TrpB located in loop B (Fig.6).

Although the key residues that shape the binding pocket are conserved in \(\alpha 7\) and muscle type receptors, a positive aminoacid lysine (153), located three residues after Trp in loop B in \(\alpha 4\), is replaced by glycine in \(\alpha 7\) or \(\alpha 1\) receptor. Lysine at position 153 is hypothesized to favor hydrogen bonding between Loop B and Loop C to shape the aromatic binding pocket, where as glycine strongly discourages this bond formation. Considerable evidence comes from the fact that a point mutation at glycine-153 with lysine (muscle type \(\alpha 1\) G153K) enhances the affinity of muscle type receptors for nicotine by altering the shape of the binding pocket. In addition to the strong cation-\(\pi\) interactions, there exists another non-covalent interaction i.e. a hydrogen bond between nicotine and the backbone carbonyl group (indicated by a star in Fig.6) of TrpB, which is weak in muscle type receptors (Xiu et al., 2009).

![Figure 7. Alignment for residues in four loops-](image)

Residues in green form the aromatic binding pocket. Notice that Lysine (K) is replaced by Glycine (G) in Loop B of \(\alpha 7\) and muscle type receptors (Blue) (Xiu et al., 2009).

### 2.3. Composition and stoichiometry of \(\alpha 4\beta 2\) receptors

\(\alpha 4\beta 2\) nAChRs are widely distributed in the brain, including cortex, midbrain, thalamus, striatum, ventral tegmental area (VTA), hippocampus, amygdala, IPN and cerebellum (Rose, 1996). These receptors are pentameric structures formed by assembly of \(\alpha 4\) and \(\beta 2\) subunits. A series of experiments conducted in Xenopus oocytes and mammalian cells by injecting different ratios of \(\alpha 4\) and \(\beta 2\) cDNAs resulted in two different populations that showed differences in agonist elicited currents accounting for two stoichiometries. The ratios of \(\alpha 4\) and \(\beta 2\) for high and low
binding sites are 2:3 and 3:2, respectively (Zwart and Vijverberg, 1998). A 100-fold difference has been reported in the EC50s for high and low affinity acetylcholine binding (1µM and 100 µM) (Penton and Lester, 2009).

The physiological functioning of nAChRs is dependent on stoichiometry and subunit composition. nAChRs are ligand-gated channels and ion permeability of these receptors is dependent on the subunit composition; homomeric α7 are highly permeable to Ca\(^{2+}\) besides Na\(^+\) and K\(^+\), whereas α4β2 exhibit relatively low permeability to Ca\(^{2+}\) (Dajas-Bailador and Wonnacott, 2004). Relevant findings suggest that α4β2 may assemble with other α-type and β-type nicotinic receptor subunits. For example, α4β2 combine with α5 in several brain regions, and with α6 or β3 or α2 subtypes in the striatum and retina (Gaimarri et al., 2007). These combinations might alter the rate of desensitization and result in a loss of upregulation (Govind et al., 2009).

2.4. α4β2 upregulation

Evidence from Positron emission tomography (PET) and post-mortem studies supports the view that chronic tobacco smoking increases the number of high-affinity binding sites for nicotine, a phenomenon known as ‘upregulation’ (Wonnacott, 1990; Wullner et al., 2008). Further, both in vivo and in vitro findings suggest an increase in number of α4β2 binding sites upon continuous exposure to nicotine (Marks et al., 1983; Whiteaker et al., 1997; Fenster et al., 1999; Buisson and Bertrand, 2001). However, the mechanism is still unclear and relatively little information is available on the protein machinery governing nAChR folding and assembly. Thus far researchers have established that it is a post-translational event and scatchard analysis indicates no change in the affinity for agonists but an increase in B\(_{\text{max}}\) (Whiteaker et al., 1998; Fenster et al., 1999; Sallette et al., 2005).

2.4.1. Agonist-induced upregulation

Nicotine acts at acetylcholine binding site and at concentrations achieved in smoker’s brain (~100-300nM) activates, desensitizes and upregulates α4β2 receptors (Fig. 8). Agonist-induced desensitization is hypothesized to trigger upregulation (Fenster et al., 1999).
Moreover, researchers have established that upregulation is not restricted to neurons, because α4β2 expressed heterologously in different culture systems including M10, HEK293, and SHEP1 elicit agonist-induced upregulation (Peng et al., 1994; Gopalakrishnan et al., 1996; Whiteaker et al., 1997; Eaton et al., 2003). Early in vivo studies conducted by Marks et al. report that nicotine administration increases the density of α4β2 receptors with no change in the message encoding for either subunits, suggesting that upregulation might be a post-translational change (Marks et al., 1983). Similarly, in vitro studies performed in distinct cell types showed no change in α4 or β2 message. Besides nicotine, other agonists that have been reported to induce upregulation are DMPP, ABT-418, epibatidine and cytisine (Gopalakrishnan et al., 1997), suggesting that upregulation maybe an intrinsic property of α4β2 receptors.

2.4.2. Antagonist elicited upregulation

α4β2 antagonists do not block but elicit upregulation themselves or potentiate agonist-induced upregulation (Wonnacott, 1990). Gopalakrishnan et al. (1997) studied the effect of DHβE on HEK293 cells stably expressing α4β2 receptors and reported that dHβE dose dependently increases [3H] cytisine binding. Moreover, this effect was not exclusive to dHβE, but other antagonists such as tubocurarine (d-TC), Mec and methyllycaconitine (MLA) produced a similar effect (Peng et al., 1994; Gopalakrishnan et al., 1997). Saturation analysis revealed that observed upregulation is due to an increase in [3H] cytisine binding and not an increase in affinity of receptors for the radioligand (Gopalakrishnan et al., 1996). Similarly, administration of dHβE in rats caused α4β2 upregulation (Govind et al.,
Synergistic effects on α4β2 upregulation are observed when antagonists are co-applied with agonists (Buisson and Bertrand, 2001). During a developmental period, chronic nicotine exposure induces significant upregulation of heteromeric nAChRs in hippocampus, cortex and thalamus without a change in mRNA expression; chronic administration of nicotine (6mg/kg/day) increased $^{125}$I-epibatidine binding compared with control animals, as shown in Fig.9B, where co-administration of nicotine and DHβE (18mg/kg/day) resulted in a stronger upregulation in the same areas of the brain (Fig.9D) (Huang et al., 2007). This unusual effect is also observed in vitro in heterologous systems expressing α4β2 receptors. Gopalakrishnan et al. (1997) showed that co-application of Mec or d-TC with nicotine significantly increases $[^3]$H cytisine binding by approximately 6-fold over controls. Moreover, Buisson and Bertrand (2001) suggest that α4β2 upregulation involves an increase in function, and both agonists and antagonists induce functional upregulation of high-affinity α4β2 nAChRs in HEK293 cells stably transfected with human α4 and β2 subunits. Thus, receptor occupancy is sufficient to trigger increases in α4β2 receptors, suggesting that any ligands binding to the receptor might be capable of eliciting upregulation regardless of channel activity.

### 2.4.3. Possible signaling pathways involved in α4β2 upregulation

In the CNS, nAChRs are localized to pre- and post-synaptic neuronal membranes and they belong to a family of ligand gated ion channels (Millar and Harkness, 2008). Acetylcholine released at the nerve terminals binds to and opens cationic channels allowing Na$^+$, K$^+$ and Ca$^{2+}$ ions through the channel (Dani and Heinemann, 1996; Dani and De Biasi, 2001). Depolarization due to Na$^+$ entry activates the voltage operated calcium channels leading to calcium influx. Ca$^{2+}$ is then released from internal stores mediated by ryanodine and IP3 receptors (Fucile, 2004; Penton and Lester, 2009). Nicotine mediates its effects through several signaling pathways based on length of exposure and activation and/or desentization of nAChRs. The intracellular increase in calcium could activate adenylate cyclase (AC), protein kinase A (PKA), PKC, calcium calmodulin kinase (CaMK) and phosphatidylinositol-3-kinase (PI3K) (Dajas-Bailador and Wonnacott, 2004; Wonnacott et al., 2006). Furthermore, these kinases phosphorylate downstream messengers such as MAPK leading to activation of transcription factor cAMP response element binding protein (CREB) (Brunzell et al., 2003; Dajas-Bailador and Wonnacott, 2004; Di Luozzo et al., 2005; Liu et al., 2007).
Activation of cAMP-dependent effectors PKA and PKC induces $\alpha_4\beta_2$ upregulation (Pollock et al., 2007; Pollock et al., 2009). Gopalakrishnan et al. (1997) reported that a membrane permeable cAMP analog, dibutyl cAMP, and a phosphodiesterase inhibitor, IBMX, elicit significant increases in $[^3]H$ cytisine binding over untreated controls. Further, co-treatment of nicotine and forskolin (adenylate cyclase stimulant) or PMA (PKC activator) caused a synergistic increase. Consistent with an increase in receptor density, these agents significantly increased acetylcholine-evoked currents. Besides $\alpha_4\beta_2$, $\alpha_7$ nAChRs also elicit upregulation; Nuutinen et al. (2006) showed that cAMP and PKC also induce $\alpha_7$ receptor upregulation in h$\alpha_7$ SHEP1 cells.

**2.4.4. Effect of temperature on $\alpha_4\beta_2$ upregulation** - Functional expression of nAChRs is temperature-sensitive; Torpedo nAChRs assemble into functional pentamers in mammalian fibroblast cells only at temperatures $27^\circ$C or lower (Claudio et al., 1987; Paulson and Claudio, 1990), although all nAChR subunits are adequately synthesized and available in the endoplasmic reticulum.

Similar to Torpedo nAChR subunits, Drosophila neuronal nAChR subunits exhibit temperature-sensitive expression. Lansdell et al. reported that Drosophila nAChRs successfully form functional receptors when transfected into mammalian HEK293 cells and grown at $\sim25^\circ$C (Lansdell et al., 1997). This temperature-sensitive expression of Torpedo and Drosophila nAChRs is intrinsic to these receptor subunits, and at temperatures higher than $25^\circ$C, these subunits misfold and fail to assemble properly despite adequate synthesis (Paulson and Claudio, 1990).
In contrast, Cooper et al. (1999) findings suggest that temperature-sensitive expression might not be intrinsic to Torpedo and Drosophila receptor subunits. Rat α4 and β2 subunits transfected in mammalian cell line TSA201 and grown at 30°C resulted in a significant increase in [3H] cytisine binding compared with cells grown at 37°C (Fig. 10). Further, this increase correlated with increases in agonist elicited elevation in intracellular calcium.

### 2.4.5. Effect of pro-inflammatory cytokines on α4β2 upregulation-

Clinical observations provide evidence for anti-inflammatory effects of nicotine against ulcerative colitis and neurological disorders (Sopori, 2002; Tracey, 2007). Substantial evidence indicates the expression of nAChRs on distinct cell types including macrophages, microglia, dendritic cells and intestinal cells (Matsunaga et al., 2001; Wang et al., 2003; de Jonge et al., 2005). Nicotine and endogenous acetylcholine suppress pro-inflammatory cytokine production (Borovikova et al., 2000; Tracey, 2002; Wang et al., 2004). An important question is whether PICs govern the expression of nAChRs for maintenance of physiological homeostasis.

Gahring et al. (2005) tested the influence of PICs IL-1β and TNFα on association of α4, β2 and β4 subunits in HEK293 cells and report that IL-1β and TNFα exposure alters the assembly of nAChR subtypes (Fig. 11). Subsequent investigation showed that TNFα when applied alone significantly enhances α4β2 upregulation as measured by [3H] cytisine binding in HEK293 cells expressing α4β2 receptors. Further, TNFα (25ng/ml) co-applied with α4β2 agonists (nicotine, cytisine, carbachol) or antagonists (dHβE) potentiated [3H] cytisine binding (Gahring et al., 2008).
Previously, researchers have established that $\alpha_4\beta_2$ upregulation does not require de novo protein synthesis (Buisson and Bertrand, 2001), however, TNF$\alpha$ elicited upregulation was blocked by actinomycin D and cyclohexamide, implicating that new protein synthesis might be involved in PIC-induced upregulation.

![Figure 11. Effect of PICs IL-1$\beta$ and TNF$\alpha$ exposure on $\alpha_4$, $\beta_2$ and $\beta_4$ nAChR assembly.](image)

*Both IL-1$\beta$ and TNF$\alpha$ induce significant upregulation of $\alpha_4\beta_2$ receptors, however only IL-1$\beta$ induces $\alpha_4\beta_4$ upregulation (Gahring et al., 2005).*

TNF$\alpha$ signaling interacts with multiple cascades including p38 MAPK (Gahring et al., 2008). A specific p38K inhibitor SB202190 decreased TNF$\alpha$ enhancement of nicotine-induced upregulation, suggesting the involvement of intracellular p38K signaling. The authors suggested that because protein synthesis was required for upregulation, p38K might be regulating the synthesis of chaperone-like proteins that facilitate assembly, folding and maturation of $\alpha_4\beta_2$ nAChRs (Gahring et al., 2008).

2.5. Proposed mechanisms for $\alpha_4\beta_2$ upregulation

Since last decade, several mechanisms have been proposed to explain upregulation of nAChRs: 1. Increase in incorporation of internal pool of receptors (Peng et al., 1994), 2. Desensitization followed by an increase in intracellular high affinity receptors (Fenster et al., 1999), 3. Enhanced intracellular maturation (Sallette et al., 2005) and 4. Ligands acting as chaperones and promoting the assembly of mature pentamers (Kuryatov et al., 2005). These mechanisms are discussed in detail below.
2.5.1. Pharmacological chaperone model-

Being cell permeant, nicotine is expected to interact with nicotine-sensitive receptor precursor at the α/β interface within endoplasmic reticulum (ER), as shown in Fig.12A.

![Nicotine as a chaperone](image)

Figure 12. Nicotine as a chaperone

A) Nicotine interacts with upregulation micro-domain at the interface between α and β subunit and favors subunit-subunit interaction (Sallette et al., 2005). B) Subunits synthesized in the ER mature and assemble to stable pentamers and are transported to surface via Golgi. Nicotine acts as maturational enhancer in ER and prevents the degradation by proteasomal system (Ancey et al., 2003; Corringer et al., 2006).

Nicotine binding to this micro-domain promotes subunit-subunit interactions and enhances folding and assembly of subunits to form a stable pentamer. Only mature pentamers are trafficked to Golgi, where sugars are trimmed, before being inserted into the cell surface (Sallette et al., 2005). Corringer et al. (2006) suggested a similar idea for nicotine induced receptor upregulation. Using pulse-chase experiments on HEK293 cells stably expressing human α4β2 receptors, they showed that the process of upregulation is initiated in the ER and nicotine acts a “maturational enhancer” to promote maturation of oligomeric precursors (Fig.12). In addition to being a chaperone, nicotine has been reported to inhibit the ubiquitin proteasome system, which could delay the removal of subunits from the ER (Rezvani et al., 2007; Rezvani et al., 2009). The above mentioned hypotheses reasonably explain nicotine-induced α4β2 receptor upregulation, however, they fail to account for antagonist- and cell impermeable agonist-elicited upregulation.

2.5.2. Conformational change model-
Whiting et al. first developed and characterized α4β2 receptors by transfecting chick α4 and β2 subunits in a mouse fibroblast cell line M10 (Whiting et al., 1991). Subsequently, Peng et al. demonstrated that up-regulation is a post-transcriptional event and antagonists do not block but synergistically potentiate nicotine-induced up-regulation (Peng et al., 1994).

Figure 13. Nicotine-Receptor subunit interaction

Similar interactions are involved when nicotine binds to unassembled subunits in the ER; Nicotine is lipophilic and acts as a chaperone to enhance assembly and maturation of nAChRs. Nicotine binding stabilizes nAChR-pentamer in the ER, which has the lowest free energy and highest stability (Nashmi et al., 2007).

b) Ligand-receptor interaction. As displayed in figure, receptors in a closed state have higher free energy, and ligand binding to receptors lowers the free energy to stabilize the interaction and also increase the affinity of ligand to the receptor. The ligand-bound desensitized receptor has the lowest free energy and highest affinity making the complex highly stable (Nashmi and Lester, 2007).

Because ion flow is not required and both permeable (nicotine, cytisine) and impermeable (DMPP) agonists induce up-regulation (Gopalakrishnan et al., 1997), a conformational change at the cell surface could lock the ligand to a high-affinity binding site and delay the degradation of receptors. At this time it was obvious that upregulation was not intrinsic to neurons, but what contributed to delayed turnover was not clear. Later, Bencherif et al. (1995) proposed the existence of an internal pool of low-affinity receptors (R⁰, undetected by radioligand), and that nicotine exposure might shift the equilibrium to a high affinity binding state (R⁻) (Bencherif et al., 1995). Nashmi and Lester (2007) suggest that channel opening leads to desensitization and upregulation may arise from desensitized receptors rather than activated receptors; the desensitized receptor state has a higher affinity for agonists compared with open and closed states; further, it has the lowest total free energy and hence it binds the agonist tightly, resulting in an overall
decrease in the dissociation constant of the drug-receptor complex (Fig.13) (Nashmi and Lester, 2007). On the other hand, several studies suggest the existence of two subpopulations of functional receptors that differ in subunit composition: high-affinity consist of \((\alpha_4)_2(\beta_2)_3\), whereas low-affinity consist of \((\alpha 4)_3(\beta 2)_2\) (Zwart and Vijverberg, 1998). A conformational change from low-affinity to a high-affinity state has been suggested to cause upregulation.

**2.5.3. cAMP-dependent phosphorylation model**

Because \(\alpha 4\beta 2\) up-regulation is a post-transcriptional event, phosphorylation or dephosphorylation of receptor subunits or proteins contributing to maturation of receptors by cAMP-dependent kinases might modulate mechanisms underlying up-regulation (Fig.14), suggesting that up-regulation may not be a mere surface phenomenon but intracellular effectors that do not interact with the receptor may also elicit up-regulation.

Activators of second messengers PKA and PKC, such as forskolin and PMA, respectively, induce significant up-regulation (Gopalakrishnan et al., 1997). Pollock et al. (2007, 2009) studied phosphorylation of mature and immature \(\alpha 4/\beta 2\) subunits in \(h\alpha 4\beta 2\) SHEP1 cells after stimulation with 10\(\mu\)M forskolin or 200nM phorbol dibutyrate (PDBu). Forskolin stimulates cAMP-dependent PKA, while PDBu stimulates PKC. These studies report that \(\alpha 4\) subunits are differentially phosphorylated by PKA and PKC; both phosphorylate immature \(\alpha 4^*\) receptors whereas only PKC phosphorylates mature \(\alpha 4^*\) receptors. Further, while both PKA and PKC increase the number of surface expressing receptors ([\(^3\)H] cytisine binding), only PKC increases receptor function. A different study reported that upregulation of \(\alpha 7\) receptors also involves cAMP-dependent PKC activation in \(h\alpha 7\) SHEP1 cells. Moreover, over-expression of recombinant PKC potentiates nicotine-induced \(\alpha 7\) binding ([\(^3\)H] MLA) compared with untransfected nicotine treated cells (Nuutinen et al., 2006).
2.5.4. ‘Ubiquitin system and ER chaperone’ model

In addition to the second messengers regulating the phosphorylation of α4 and β2 subunits, the ubiquitin proteasome system is suggested to regulate the availability of nAChR subunits (Christianson and Green, 2004; Rezvani et al., 2007). For instance, Ficklin et al. (2005) found that a ubiquitin-like protein, Ubiquilin 1, interacts with unassembled α4 nAChR subunits and suggest that it might regulate the expression of α4β2 nAChRs. Co-immunoprecipitation studies in mouse brain lysates suggest that ubiquilin-1 interacts specifically with α4, α3 and β4 subunits. In the same study, lentiviral over-expression of ubiquilin-1 in cultured superior cervical ganglia (SCG) neurons significantly decreased nicotine-induced upregulation of α3* nAChRs. Although this implicates a negative regulatory function of this protein in maturation of nAChRs, it is not clear yet whether nicotine exposure alters the message or protein for ubiquilin-1 (Ficklin et al., 2005). The domain structure of ubiquilin-1 is shown in Figure 15.
a) ERP57 and CALNEXIN- ER chaperones ERP57, calnexin and BiP are reported to promote assembly, delay endoplasmic reticulum associated degradation and aid in removal of misfolded proteins (Wanamaker and Green, 2007). In a recent study, muscle nAChR subunits were shown to interact with ER chaperones ERP57 (a thiol-disulfide oxidoreductase), calnexin (CN) and immunoglobulin binding protein (BiP) (Wanamaker and Green, 2007). These proteins were suggested to play a role in promoting assembly, delay endoplasmic reticulum associated degradation (ERAD) and also aid in removal of misfolded proteins (Wanamaker and Green, 2007; Millar and Harkness, 2008). The interaction of BiP with receptor subunits is short-lived owing to its dependence on ATP, whereas the interaction of subunits with ERP57 and CN was long-lived. Consistently, over-expression of CN significantly increased the expression of nAChRs, perhaps by delaying ERAD (Wanamaker and Green, 2007).

b) CRELD2: Cysteine Rich EGF-like domain 2- Recently, using yeast two hybrid method, a novel ER stress-inducible gene CRELD2 was discovered by Manuel Criado’s laboratory in Spain. Ortiz et al. (2005) suggest that CRELD2 regulates α4/β2 nAChR expression in Xenopus oocytes. They showed that co-expression of CRELD2 α/β with α4/β2 significantly reduces agonist evoked current amplitudes in Xenopus oocytes, suggesting a negative association between α4β2 and CRELD2 expression. Six isoforms of CRELD2 have been identified so far; CRELD2α, β, γ, δ, ε and ζ (Maslen et al., 2006). A schematic of protein domains and amino acid sequence for CRELD2α and β are shown in Figure 16.

Using microarray analysis of thapsigargin-inducible genes, Oh-hashi et al. identified CRELD2 gene in Neuro2a cells as a novel ER-stress inducible gene. Using myc-tagged CRELD2 constructs, they report that it is localized to ER and golgi, and the CRELD2 promoter region has a consensus endoplasmic reticulum stress element (ERSE) site, which could be activated during unfolded protein accumulation by ER stress sensors such as PERK, IRE and ATF6 (Oh-hashi et al., 2009).
c) HERPUD1/HERP Homocysteine-inducible endoplasmic reticulum stress protein.

This ER localized protein has an intracellular N-terminal Ubiquitin-like domain (Fig.17), which could play a prominent role in post-transcriptional modifications, targeting misfolded proteins to proteasome system (ERAD) and assembly and stabilization of proteins (Kokame et al., 2000). In addition, the promoter region has c/EBP-ATF and ERSE sites that are regulated by both ER stress and cellular stress pathways (Ma and Hendershot, 2004).
The mammalian unfolded protein response (UPR) activates PERK, ATF6 and Ire1 signal transducers, which activate downstream effectors to initiate gene induction during stress (Fig.18). Under normal physiology, these three signal transducers are inactive by being bound to BiP. ER and other cellular stresses initiate BiP dissociation leading to translocation of ATF6 and Ire1 to the nucleus. Dissociated PERK phosphorylates another effector, eIF-2α, which in turn induces ATF4 expression (Fig.18). While ATF6 and Ire1 bind to ERSE sites in the promoter region, ATF4 binds to ATF-C/EBP composite sites to initiate gene transcription (Ma and Hendershot, 2004).

Figure 17 HERP

Hydropathic profile of amino acid sequence of HERP. Kokame et al. (Kokame et al., 2000) suggest that the majority of the HERP molecule faces the cytoplasm.

Figure 18. HERP has both ERSE and ATF-C/EBP sites.

HERPUD1 has both ERSE and ATF-C/EBP sites in the promoter region, making it a target for regulation by both PERK and ATF6/Ire1 signal transducers. HERP is regulated not only by cellular stressors such as amino acid deprivation, viral infection, heme deficiency etc through activation of the PERK pathway, but also by ER stressors through the ATF6/Ire1 pathway (Kokame et al., 2000; Ma and Hendershot, 2004).
d) **Ric3**: An ER chaperone Ric3, Resistant to inhibitors of cholinesterase 3, is a transmembrane protein that interacts with unassembled nAChR subunits and aids in their assembly and maturation (Castelan et al., 2008; Millar, 2008; Wang et al., 2009). Research suggests that Ric3 is essential for functional expression of α7 nAChRs (Castillo et al., 2006; Lansdell et al., 2008). For instance, in α7 non-permissive cell lines, co-expression with Ric3 allows assembly of subunits to full pentamers and in turn functional expression of α7 nAChRs (Lansdell et al., 2005). Although transient Ric-3 transfection enhances nAChR expression, other factors seem to influence Ric-3 function. For example, a recent study (Lansdell et al., 2008) suggests that Ric-3 chaperone activity is host cell dependent because Ric-3 showed differential activity when expressed in either a Drosophila or a human cell line.

**Figure 19. RIC3 protein domains**

- a) Predicted domains of Ric3 protein in humans and invertebrates *C. elegans* and *Drosophila*. 
- b) Predicted topology of Ric3. The N-terminal signal sequence is hypothesized to be cleaved in vertebrates resulting in a single transmembrane domain, whereas invertebrates have two transmembrane domains (Millar, 2008).

Ric3’s role on α4β2 expression is rather contradictory. In Halevi et al. and Castillo et al., co-expression of hRic-3 in oocytes expressing α4β2 receptors, significantly reduced the current amplitude compared with no Ric-3 controls (Halevi et al., 2003; Castillo et al., 2006). In contrast, Lansdell et al. show that co-expression of hRic3 and α4β2 receptors in human cells (tsA201) significantly increases specific [³H]epibatidine binding (Lansdell et al., 2005). The mechanism by which Ric3 influences nAChR expression is still unclear. The presence of hydrophobic amino acids at position 202 makes α4β2 receptors a target for inhibition by hRic3. α3β4 are only partially inhibited because though β4 has an isoleucine at 202, α3 has a polar tyrosine at this position (Millar, 2008; Treinin, 2008). Sequence homology
studies suggest that Ric3 has two transmembrane domains (Fig.19), both termini located in the cytoplasm. Experimental evidence indicates that N-terminal signal sequence of human Ric3 is cleaved resulting in a single transmembrane domain (Millar, 2008).

3. αβ2 Upregulation and Nicotine addiction

Nicotine, a tobacco plant alkaloid, interacts with αβ2 nAChRs in the CNS. Nicotine fails to change brain dopamine levels in α4 or β2 knock-out (KO) mice, and additionally KO mice do not self-administer nicotine (King et al., 2004), suggesting that αβ2 nAChRs are involved in nicotine-induced rewarding and reinforcing behavior (Tapper et al., 2004). Evidence from studies in non-human primates and other rodent models reveals that drugs of abuse including cocaine and amphetamine enhance dopamine (DA) release in the nucleus accumbens (NAcc) (Groves and Rebec, 1976; Di Chiara and Imperato, 1988; Cornish and O'Brien, 1996; Kauer, 2004). Likewise, nicotine mediates pleasure by increasing DA levels in the NAcc (Benowitz, 2009). However, at 100-300 nM concentrations after the first cigarette puff, nicotine desensitizes most nAChRs on pre-synaptic and DAergic neurons (Fig.20), yet nicotine continues to elevate DA levels in NAcc to a ‘mM’ range, measured using cyclic voltammetry (CV) in mouse cortical slices (Yu et al., 2004). Desensitization of nAChRs is hypothesized to attenuate DA release and to produce tolerance (Dani and Heinemann, 1996; Dani and De Biasi, 2001; Gentry and Lukas, 2002). Zhang and Sulzer (2004) supported the idea that desensitization of nAChRs attenuates DA release, and proposed a theory of ‘frequency dependent modulation’ of DA release by nicotine to explain why nicotine continues to elevate DA levels, though receptors are desensitized. DAergic neurons projecting to ventral striatum fire action potentials tonically below <10 Hz but a higher frequency (100 Hz) enhances DA release even when the receptors are desensitized (Fig.20). Investigators have studied the firing of DAergic neurons in brain slices and demonstrate that nicotine, similar to cocaine and amphetamine, shifts the firing of DAergic neurons from spontaneous, tonic, low frequency causing ‘µM’ DA release to phasic, high-frequency bursting causing ‘mM’ DA release (Mansvelder et al., 2002; Rice and Cragg, 2004; Zhang and Sulzer, 2004; Goto and Grace, 2007).

In addition, Zhang and Sulzer (2004) found that desensitization of nAChRs disrupts tonic DA release but not phasic - A single low pulse stimulus elicited a maximum dopamine concentration of 0.66± 0.11µM, which was blocked in the presence of 300 nM nicotine or 2 µM mecamylamine, whereas a higher stimulation frequency (20-100

20
Hz) significantly elevated DA release, which was not blocked by either drug. But how the drugs of abuse switch the frequency mode from tonic to phasic is still an emerging field. (Recent studies report that genetic inactivation of the essential NR1 subunit of NMDA receptors in DAergic neurons in the VTA disrupts acquisition of behavioral responses and also memory encoding (Zweifel et al., 2009), implicating a role for NMDA receptors in facilitating a switch in firing frequency).
Does α4β2 Up-regulation add fuel to the fire? Tobacco smoking events encode specific memory patterns in the brain that are activated by smoking-related cues, and this memory pattern is associated with pleasurable sensations and reward that regulate smoking behavior. A long standing question has been whether upregulation plays a role in nicotine-induced effects. Chronic tobacco smoking induces a cell-specific α4β2 up-regulation in the CNS (Marks et al., 1983; Schwartz and Kellar, 1983; Nguyen et al., 2003). For instance, as displayed in Figure 20, though α4β2 receptors are well distributed on DAergic, excitatory glutamatergic and inhibitory GABAergic neurons of the VTA, chronic nicotine selectively upregulates α4β2 on glutamatergic and GABAergic neurons (Tapper et al., 2004; Nashmi et al., 2007), but not on DAergic neurons.

Nicotine induces long term potentiation (LTP) in acute nicotine-primed rats (Hamid et al., 1997), and upregulation of α4* nAChRs on glutamatergic neurons lowers the threshold for long term potentiation (Nashmi et al., 2007). Therefore, nicotine-induced upregulation might synergistically stimulate excitatory inputs onto DAergic neurons to facilitate activation of NMDA receptors (Fig.20) and initiate chronic smoking-associated addiction, tolerance and cognitive sensitization (Tapper et al., 2004; Nashmi et al., 2007; Penton and Lester, 2009). Strong evidence comes from a recent study demonstrating that upregulation of α4* nAChRs in the GABAergic interneurons increases the inhibitory effect on DAergic neurons in the VTA, and this contributes to tolerance (Nashmi et al., 2007). Thus, α4β2 upregulation might regulate nicotine-induced rewarding and reinforcing behavior by altering the physiology of excitatory and inhibitory neurotransmission.

4. Objectives and specific aims

To establish screening paradigm for finding genes altering surface α4β2 expression-

Treatment for smoking cessation requires a thorough understanding of α4β2 upregulation, and the underlying mechanism is still unclear. Recent studies implicate the role for ER chaperones and ubiquitin proteasome system in regulating the folding, assembly and maturation of α4β2 nAChRs (Rezvani et al., 2007; Wanamaker and Green, 2007; Millar, 2008; Rezvani et al., 2009). In addition, cAMP-dependent phosphorylation of α4 and β2 subunits induces upregulation (Pollock et al., 2007; Pollock et al., 2009). All of the protein machinery involved in regulating the folding, assembly, maturation and trafficking of α4β2 receptors is far from being clear. This work will help identify and clarify the role of some of these proteins in regulating α4β2 surface receptor expression. As a part of the this study,
we identified and tested the function of genes influencing α4β2 receptor expression in hα4β2 SHEP1 cells. The specific aims were:

1. **To validate microarray analysis of nicotine-induced upregulation using quantitative RT-PCR.**

   We used a microarray-based approach to investigate whether nicotine modulates gene expression while inducing up-regulation of α4β2 receptors, with special interest in ER chaperones and other proteins that promote assembly, folding, and/or receptor trafficking. We investigated the effects of 10µM nicotine on gene expression at 0.25h, 1h, 8h and 24h. Quantitative RT-PCR validated most of the predicted gene expression changes. [³H] cytisine binding assays were performed on sister cultures to ensure that cells showed significant receptor up-regulation.

2. **To screen for genes that influence α4β2 up-regulation.**

   Consistent with previous literature, our preliminary data suggested that a competitive antagonist, dHβE and a channel blocker, Mec, elicit upregulation or potentiate nicotine-induced upregulation (Gopalakrishnan et al., 1997). Our working hypothesis was that because antagonists potentiate α4β2 upregulation, the expression of genes influencing upregulation must show a correlation (positive or negative) with both nicotine-induced as well as antagonist-potentiated upregulation. To test this hypothesis, we screened for ER chaperones CRELD2, HERPUD1, PDIA6, PDIA4 and BiP, and a transcription factor CREM, observed to be altered during microarray analysis, using a correlation-analysis approach.

3. **To investigate whether α4β2 upregulation can be influenced by manipulating the expression of genes identified in Aim #2.**

   A preliminary screen by correlation analysis involving six gene transcripts identified two of six as possible candidates (CREM and CRELD2). We used RNA interference and a non-viral over-expression method to manipulate CRELD2 expression, as an example, in hα4β2 SHEP1 cells.

**Significance:** The results of this study may aid in advancing our understanding of α4β2 upregulation and also designing better therapeutics for smoking cessation. α4β2 upregulation influences nicotine-induced detrimental effects including reinforcing behavior and tolerance. Traditional pharmacological methods using antagonists to block upregulation have failed because they also elicit or potentiate nicotine-induced upregulation, limiting the research tools
available to investigate this phenomenon. Correlation analysis coupled with gene manipulation would be an ideal way of approaching this issue to not only identify the chaperones but also ask the relevant function of individual genes contributing to upregulation.

**Study design.** Schematic below represents the study design that involved two screening processes after the microarray analysis: 1. correlation analysis and 2. siRNA-mediated gene silencing. We established a screening paradigm for identification and testing the function of genes contributing to α4β2 upregulation.

![Schematic of study design](image)

**Screen 1**
- **Correlation Analysis**
  - Identifying genes: CRELD2, CREM, TMEM50B etc
  - Asking gene function

**Screen 2**
- **RNA interference**
  - Silencing CRELD2 increases α4β2 receptors
  - Selection of siRNA: siRNA duplex targeting a single gene
  - Optimization of Transfection:
    1. Different transfection reagents
    2. SiRNA Dose Response Curve (1nM, 3nM, 30nM, 100nM)
    3. Time required for maximum silencing (24h, 48h and 72h)
  - Transfection Efficiency:
    1. mRNA levels using qPCR (GAPDH as a positive control)
    2. Scrambled or mismatch siRNA as a negative control
5. Materials and methods

Cell culture- SHEP1 human neuroblastoma cells stably transfected with human α4 and β2 subunits (a generous gift from Ron Lukas) were grown at 37°C in 5% CO₂ as recommended (Pacheco et al., 2001). Cells grown in 75 cm² or 150 cm² flasks were passaged onto 6-, 24- or 96-well plates and grown for two to three days to attain confluence. Cells were then treated with (-)-nicotine or (-)-nicotine plus dHβE or Mec for indicated times before harvesting.

Radioassay of surface α4β2 receptors- Cells grown in 6-well or 24-well plates were treated with/without (-)-nicotine or (-)-nicotine plus dHβE or Mec for varying times. After incubation, cells were washed three times with ice cold PBS and incubated with 10nM [3H]-cytisine for 75 min at 4°C. Cells were then washed three times with 1ml ice cold HBSS (Sigma Aldrich) and suspended in extraction buffer (0.1 M NaOH, 1% Triton X-100) for 10 min on a shaker. Radioactivity was measured using a Beckman Scintillation counter. Non specific binding was determined in presence of 20μM (-)-nicotine. When binding assays were performed on beads, the conditions were similar, but done in a total volume of 100 μl in PBS-Triton (100 mM NaCl, 10 mM NaH₂PO₄, pH 7.4, 0.5% Triton X-100), followed by 3 x 1ml PBS-Triton washes before using the extraction buffer.

Total RNA extraction and quantitative Real Time PCR- In separate experiments from the microarray analysis, total RNA was extracted from cells grown in 6-well or 24-well plates using the Absolutely RNA miniprep kit (Stratagene). Briefly, after adding lysis buffer cells were flash frozen and stored (not more than 10 days) at -20°C for later use, or immediately proceeded for mRNA purification. Purified RNA was quantified by use of a NanoDrop® ND-1000 UV-Vis Spectrophotometer. 200ng of total RNA from each sample was reverse transcribed by Transcriptor First Strand cDNA synthesis system (Roche Applied Science). qPCR was performed using FastStart SYBR green Master (ROX) (Roche) and an ABI 7000 thermocycler. PCR amplification involved a pre-cycle at 95°C for 10 min followed by 40 cycles of a denaturation step at 95°C for 15 seconds and then 60°C for one minute and 72°C for 30 seconds. cDNA amplification products were analyzed by agarose gel electrophoresis and sequenced to confirm the amplicons. Changes in mRNA were analyzed by the comparative (ΔΔ C_T) method (User Bulletin #2: ABI Prism 7700 Sequence Detection System) and normalized against GAPDH as an internal control. Primers (Table 3) were designed using IDT SCiTools PrimerQuest™ or Primer3 (Rozen and Skaletsky, 2000), and purchased from Integrated DNA Technologies (IDT).

Microarray Analysis and “Retrospective” Nicotinic Stimulation Experimental Design- Total RNA was processed for analysis on Affymetrix HG-U133A human genome probe arrays following the manufacturer’s protocol. Briefly,
cDNA template was generated from T7/oligo dT (Affymetrix) primed RNA. Purified cDNA template was then used to create amplified, biotin labeled, antisense cRNA through in vitro transcription using T7 RNA Polymerase. Labeled cRNA was quantified and added to a hybridization cocktail ready for hybridization to an Affymetrix HG-U133A. Probe arrays were hybridized and processed according to the Affymetrix Expression Analysis Manual. Data was generated using a GCS 3000 Scanner with attached Autoloader, run with GeneChip Operating System 1.1.1 (GCOS, Affymetrix). Raw image files were uploaded into Stratagene ArrayAssist software searching for statistically significant concurrent changes within each time point and across duplicate experiments. Transcripts of interest for each time point were further interrogated using Pathway Architect (Both software packages now contained within Agilent GeneSpring version 10.0) for biological relatedness. In preliminary experiments, merely changing the medium caused significant changes in over 1500 gene transcripts for untreated hα4β2 SH-EP1 cells, compared to over 900 changes induced by medium change in untreated wild-type SH-EP1 cells. These non-specific gene changes threatened to mask any effects due to nicotinic stimulation. Therefore, a “retrospective” nicotinic stimulation design minimized these non-specific effects. All cells were harvested for RNA at the same time to minimize differences in cell confluency or the number of divisions since plating. Human α4β2 SH-EP1 cells were seeded in 6 well plates in triplicate at a density of approx. 5x10⁴ cells per well 48h prior to harvest, and media changes brought the nicotine concentration to 10µM at 24, 40, 47 and 47.75 h after plating (corresponding to 24, 8, 1 or .25 h prior to when the cells were harvested). Quadruplicate sister cultures in 24 well plates seeded at a density of approx. 1x10⁴ cells per well were treated similarly at the indicated times and assayed for [³H]-cytisine binding to determine the amount of nicotine-induced up-regulation.

**RNA interference**- siRNA duplex targeting CRELD2 was purchased from Sigma Aldrich (SASI_Hs01_00027506). The siRNA duplex was transfected into the cells grown to ~60% confluence, using X-tremeGene siRNA transfection reagent (Roche). The knockdown efficiency was measured by qPCR. To determine the influence of CRELD2 on α4β2 up-regulation, cells seeded in 24-well dishes were transfected with 30nM siRNA for 48hrs and treated with or without 10µM nicotine for 24hrs. Following incubation, surface receptor expression was determined by [³H]-cytisine binding assays. Effects of Mec and dHβE on CRELD2 expression were measured using qPCR in wild-type or hα4β2 SH-EP1 cells.
**Data analyses** - SPSS 15 and GraphPad Prism were used for data analysis. To assess significant differences between control and drug-treated samples, Student’s t-test was performed. One-way analysis of variance (ANOVA) was used for comparisons among groups followed by Post-hoc tests (Tukey).

### 6. Results

#### 6.1. Nicotine-induced upregulation of α4β2 nAChRs

Exposing SHEP1 human neuroblastoma cells stably transfected with human α4 and β2 subunits (hα4β2 SHEP1 cells) (Pacheco et al., 2001) to 10µM nicotine for 24h increased the receptors in a concentration-dependent manner. This nicotine concentration produced the maximum up-regulation of the concentrations tested (Fig.21A).

10µM (-)-nicotine required more than 5-6h to induce upregulation in hα4β2 SHEP1 cells (Fig.21B), whereas wild-type cells, lacking α4β2 receptors, showed no specific [3H] cytisine binding. The magnitude of upregulation varied from one and a half to three-fold among experiments. Experiments were repeated three times, with four biological replicates. Binding assays on sister cultures confirmed α4β2 receptor upregulation when mRNA was harvested for microarray analysis or qPCR.

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**Figure 21. α4β2 upregulation in hα4β2 SHEP1 cells.**

A) Dose-dependence of nicotine-induced upregulation observed in hα4β2 SHEP1 cells. The lowest dose to produce maximum upregulation was 10µM. B) Time-course nicotine on hα4β2 SHEP1 cells. Wild type and hα4β2 SHEP1 cells seeded onto 24-well plates were exposed to 10µM nicotine for indicated times and specific binding was determined. 20µM nicotine was used to estimate non-specific binding. Results represent mean ± SD of three independent experiments in quadruplicate.
6.2. Microarray analysis of nicotine-induced α4β2 upregulation

The microarray analysis (performed by Scott Leppanen and Adham Abutaha) was validated using quantitative RT-PCR. The hα4β2 transfected SHEP1 cells were treated with 10µM (-)-nicotine for 1h, 8h and 24h. Post treatment cells were harvested and mRNA isolated was reverse transcribed into cDNA for quantitative RT-PCR analysis. 10µM nicotine significantly altered transcripts for 41 probe sets on Affymetrix HG-U133A arrays measured at 0.25h, 1h, 8h or 24h exposure, with 8h showing the maximum number of altered genes (Tables 1 & 9). However, nicotine did not change expression in the α4 probe set at the time points tested (The Affymetrix β2 probe set consists of untranslated regions, and could not measure expression changes in the β2 open reading frame used in hα4β2 SHEP1 cells. However, qPCR confirmed that nicotine caused no change in either α4 or β2 mRNA expression (Table 2).

Table 1. Possible endoplasmic reticulum chaperones and miscellaneous genes

ER chaperones (*), transcription factors (#), cytosolic and other unrelated gene transcripts altered by 10µM nicotine in hα4β2 SHEP1 cells in microarrays compared with untreated controls. (Categorized by Pathway Architect™ (Stratagene)).

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene ID</th>
<th>Gene title</th>
<th>Accession #</th>
<th>Time point</th>
<th>Fold Change (p&lt;0.005)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARMET</td>
<td>7873</td>
<td>Arginine-rich, mutated in early stage tumors</td>
<td>NM_006010</td>
<td>8h</td>
<td>1.448</td>
</tr>
<tr>
<td>BLCAP</td>
<td>10904</td>
<td>Bladder cancer associated protein</td>
<td>NM_006698</td>
<td>0.25h</td>
<td>0.826</td>
</tr>
<tr>
<td>BNIP3</td>
<td>664</td>
<td>BCL2/adenovirus E1B 19kDa interacting protein 3</td>
<td>NM_004052</td>
<td>8h</td>
<td>0.888</td>
</tr>
<tr>
<td>*TMEM50B</td>
<td>757</td>
<td>Transmembrane protein 50B</td>
<td>NM_006134</td>
<td>8h</td>
<td>1.823</td>
</tr>
<tr>
<td>CDYL</td>
<td>9425</td>
<td>Chromodomain protein, Y-like</td>
<td>NM_004824</td>
<td>8h</td>
<td>0.839</td>
</tr>
<tr>
<td>COL4A1</td>
<td>1282</td>
<td>Collagen, type IV, alpha 1</td>
<td>NM_001845</td>
<td>8h</td>
<td>0.732</td>
</tr>
<tr>
<td>*CRELD2</td>
<td>79174</td>
<td>Cysteine-rich with EGF-like domains 2</td>
<td>NM_024324</td>
<td>8h</td>
<td>1.502</td>
</tr>
<tr>
<td>#CREM</td>
<td>1390</td>
<td>cAMP responsive element modulator</td>
<td>NM_001881</td>
<td>24h</td>
<td>1.542</td>
</tr>
<tr>
<td>CTBP2</td>
<td>1488</td>
<td>C-terminal binding protein 2</td>
<td>NM_022802</td>
<td>8h</td>
<td>1.583</td>
</tr>
<tr>
<td>ETKN1</td>
<td>55500</td>
<td>Ethanolamine kinase 1</td>
<td>NM_018638</td>
<td>1h</td>
<td>0.857</td>
</tr>
<tr>
<td>*FKBP11</td>
<td>51303</td>
<td>FK506 binding protein 11, 19 kDa</td>
<td>NM_016594</td>
<td>8h</td>
<td>1.285</td>
</tr>
<tr>
<td>GBP1</td>
<td>2633</td>
<td>Guanylate binding protein 1, interferon-inducible, 67kDa</td>
<td>NM_002053</td>
<td>1h</td>
<td>1.234</td>
</tr>
</tbody>
</table>
6.3. Validation of transcript changes using qPCR

We selected the following gene transcripts (Table 2) for validation: Transcription factors CREM and PAX5; and 3) ER resident proteins such as BiP (HSPA5), PDIA4, PDIA6, CRELD2 and HERPUD1. Primers (Table 3) were designed using Integrated DNA Technology SciTools or Primer3 software (Rozen and Skaletsky, 2000).

Table 2. Gene transcripts selected for validation by qPCR.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Accession #</th>
<th>mRNA fold change by qPCR (10μM Nicotine exposure)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRELD2</td>
<td>NM_024324</td>
<td>1.28±0.20 1.46±0.08* 1.37±0.20*</td>
</tr>
<tr>
<td>HERPUD1</td>
<td>NM_001010990</td>
<td>1.01±0.12 1.36±0.04* 0.86±0.12</td>
</tr>
<tr>
<td>PDIA6</td>
<td>NM_005742</td>
<td>0.97±0.04 1.38±0.11* 1.08±0.07</td>
</tr>
<tr>
<td>*PDIA4</td>
<td>NM_004911</td>
<td>0.98±0.03 1.12±0.09 1.01±0.08</td>
</tr>
<tr>
<td>*HSPA5 (BiP)</td>
<td>NM_005347</td>
<td>1.00±0.10 0.91±0.01 0.98±0.03</td>
</tr>
<tr>
<td>CREM</td>
<td>NM_001881</td>
<td>1.01±0.05 1.28±0.01 1.42±0.08*</td>
</tr>
<tr>
<td>*PAX5</td>
<td>NM_016734</td>
<td>1.00±0.07 0.94±0.05 1.01±0.08</td>
</tr>
<tr>
<td>CHRNA4 (α4)</td>
<td>NM_000744</td>
<td>1.01±0.04 0.87±0.08 1.01±0.04</td>
</tr>
<tr>
<td>CHRN2B (β2)</td>
<td>NM_000748</td>
<td>1.03±0.02 1.12±0.15 1.13±0.12</td>
</tr>
</tbody>
</table>

a Gene transcripts predicted by microarray but not validated by qPCR.

* Significant at 0.05 level (n=3 with quadruplicate readings).
We considered several housekeeping genes for comparison, such as HPRT1, PMCA4 and β-Actin, before selecting GAPDH as the most appropriate endogenous control for qPCR (Calcagno et al., 2006) on the basis of its minimal Ct (thermal cycles) variation (< ± 0.15 Ct) across experimental conditions, as well as being expressed at levels similar to the transcripts under investigation (Fig.22).

As predicted, nicotine increased the mRNA levels of PDIA6 (8h only), CRELD2 (8 and 24h), and HERPUD1 (8h). The cAMP response element modulator (CREM) showed a significant delayed induction after 24h nicotine exposure. In addition, genes such as TNF-AIP3, BLCAP, GBP1 and IER3 also exhibited alterations post nicotine treatment (not shown). In contrast, qPCR did not validate transcript changes predicted by the microarray for BiP, PDIA4, and PAX5 (Table 2). Continuous nicotine exposure to wild type SHEP1 cells did not show any significant alterations in these transcripts, suggesting that the effects are mediated through α4β2 receptors.

**Table 3. qPCR primers**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT1</td>
<td>5' - AGG ACT GAA CGT CTT GCT GCA GAT - 3'</td>
<td>5' - ACT GCC TCA CCA AGG AAA GCA AAG- 3'</td>
</tr>
<tr>
<td>PMCA4</td>
<td>5' - TGC ATA GCT TAC CGG GAC TTC GAT - 3'</td>
<td>5' - GCG GAT GAG CCG GTT GAA TTC TTT - 3'</td>
</tr>
<tr>
<td>β actin</td>
<td>5' - GGC CGA GGA CTT TTA TTG CAC ATT - 3'</td>
<td>5' - GGG CAC GAA GGC TCA TCA TCA AAA - 3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5' - TTC GAC AGT CAG CCG CAT CTT - 3'</td>
<td>5' - ACC AAA TCC GTT GAC TCC GAC CTT - 3'</td>
</tr>
<tr>
<td>Alpha 4</td>
<td>5' - AAA TGC ACA TGC AAG AAG GAG CCC - 3'</td>
<td>5' - TCA GTT GTT CTG CAA TGT ACT GGA - 3'</td>
</tr>
<tr>
<td>Beta 2</td>
<td>5' - ATG ACT TCA TCA TTC GCC GCA AGC - 3'</td>
<td>5' - ACA ACG TCA TCT TCT CGC CAC AGT - 3'</td>
</tr>
<tr>
<td>CREM</td>
<td>5' - GCA GCA ATT GCA GAG ACA GAT G - 3'</td>
<td>5' - GCC ACA TCA GAG GAC AGT TCA TTC - 3'</td>
</tr>
<tr>
<td>PAX5</td>
<td>5' - TCC TGG TTT CTT CCT GCC TTC ATT TCT - 3'</td>
<td>5' - TCC AGC CCT CAC ATT CAA AGT CCA - 3'</td>
</tr>
<tr>
<td>HERPUD1</td>
<td>5' - TGA AAC TGA AGA CCC CAA CC - 3'</td>
<td>5' - CCT CCA ACA AGT ACA GCA CA - 3'</td>
</tr>
<tr>
<td>CRELD2</td>
<td>5' - CTT CTC TCC AGG AAC CTA CG - 3'</td>
<td>5' - GTC TT GCA GGA GTC GTC ACA - 3'</td>
</tr>
<tr>
<td>PDIA6</td>
<td>5' - TGC TGC TTG AAT GTT CTT GG - 3'</td>
<td>5' - GTT TGT GGG GAG GAA AGA CA - 3'</td>
</tr>
<tr>
<td>HSPA5</td>
<td>5' - TAG CTT ATG GTG CTG CTG TC - 3'</td>
<td>5' - TTT GTC AGG GTT CTT TCA CC - 3'</td>
</tr>
<tr>
<td>PDIA4</td>
<td>5' - CAT CAA GGA CTT CGT GCT GA - 3'</td>
<td>5' - TTC ACC TCC CCA GCA TAG TC - 3'</td>
</tr>
</tbody>
</table>

†primers designed by Primer3 software.
Cell Viability assays. In an attempt to investigate whether cell death was contributing to down regulation of genes, the sulforhodamine B assay (Vichai and Kirtikara, 2006) was performed. Transfected SHEP1 cells seeded onto 24 well plates were grown to sub-confluence for 2 days and then treated with 10µM (-)-nicotine for indicated time points. In comparison with untreated control cells, nicotine-treated cells did not show any significant change in cell viability (Fig.23). In addition, there was no change in the morphology of the cells (Fig.23). These findings suggest that cell death is not contributing to decreases in mRNA levels of gene transcripts and therefore, chronic (-)-nicotine exposure is inducing these gene alterations in transfected SHEP1 cells.

Figure 23. Sulforhodamine B assay.

Cells grown to confluence were exposed to 10µM nicotine for indicated times. Untreated cells served as controls. Data are means from three different experiments. Error bars represent mean±SEM

6.4. Antagonists potentiate nicotine-induced upregulation

Previous studies demonstrate that up-regulation of α4β2 receptors requires receptor occupation, but not necessarily ion flux through the receptors (Gopalakrishnan et al., 1997; Darsow et al., 2005). In addition, rather than blocking agonist-induced up-regulation, nicotinic antagonists may either elicit up-regulation themselves or potentiate agonist-induced upregulation. In an attempt to determine the dose of antagonists required to antagonize nicotine induced effects, dose response inhibition curves of [3H] cytisine binding were performed. Of the two antagonists selected, dHβE dose dependently inhibited [3H] cytisine specific binding whereas Mec, a non-competitive channel blocker, exhibited no inhibition (Fig.24). After exposure of cells with either 100µM dHβE or 10µM Mec for 48 hours, [3H] cytisine binding was increased by ~1.5 and ~2 fold, respectively. However, co-treatment of SHEP1 hα4β2 cells with nicotine and Mec or dHβE resulted in ~2.5 and ~3 fold increases in [3H] cytisine binding, respectively, compared with untreated
Figure 24. Dose response inhibition curves

Dose response inhibition of [\textsuperscript{3}H] cytisine binding by antagonists Mec and dH\textbeta E.
Results represent mean±sem.

Figure 25. Antagonists potentiate nicotine-induced α\textbeta2 up-regulation.

(A) Cells grown in 96 well plates were incubated with 100µM dH\textbeta E alone or a combination of 10µM nicotine plus 100µM dH\textbeta E for indicated times before estimating specific \textsuperscript{3}H cytisine binding. Specific basal binding was approximately 270 CPM. Co-application resulted in a ~2 fold increase in surface receptor binding. (B) Exposing cells to 10µM Mec or 10µM nicotine plus 10µM Mec for indicated times resulted in a significant increase in specific \textsuperscript{3}H-cytisine binding after 8h and 24h exposure. Experiments were in triplicate with four replicates. Results represent mean ± SD. Nic (\#P<0.05); Nic+ dH\textbeta E or Mec (*P<0.05, **P<0.01).
controls. In addition, both the treatments (nicotine+ dHβE or Mec) induced significant up-regulation in a time-dependent manner as depicted in Figure 25. These data are consistent with previous reports that up-regulation of human α4β2 nAChRs does not require channel activity or receptor activation (Gopalakrishnan et al., 1997). Since Mec and dHβE synergistically enhance up-regulation effects of nicotine, we expected that both antagonists should have a synergistic effect (either positive or negative) on genes that might be directly involved in nicotine-induced receptor up-regulation. However, both antagonists blocked nicotine-mediated gene alterations of the ER chaperones tested (Table 4). Interestingly, we observed a strong negative correlation of CRELD2 mRNA with α4β2 receptor expression (Pearson correlation, -0.813; p<0.05). In addition, CREM mRNA displayed a positive correlation (Pearson correlation, 0.990; p<0.05). Collectively, these data suggest that nicotine-mediated gene alteration of ER chaperones requires activation of α4β2 nAChRs, and based on correlation analysis, manipulation of mRNA levels of CRELD2, and CREM may influence α4β2 receptor expression. Since nicotine-induced gene alteration of CREM lagged the others and appeared after α4β2 up-regulation (24hrs), we only assessed the influence of CRELD2 on surface expression of α4β2 receptors, using RNA interference.

Table 4. Effect of antagonists dHβE and Mec on nicotine-induced gene alterations

<table>
<thead>
<tr>
<th>mRNA (fold change)</th>
<th>CRELD2(8h)</th>
<th>CREM(24h)</th>
<th>HERP(8h)</th>
<th>PDIA6(8h)</th>
<th>BiP(8h)</th>
<th>PDIA4(8h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.02±0.02</td>
<td>1.01±0.20</td>
<td>1.00±0.02</td>
<td>1.02±0.02</td>
<td>1.01±0.12</td>
<td>1.01±0.03</td>
</tr>
<tr>
<td>Nicotine</td>
<td>1.26±0.08*</td>
<td>1.36±0.06*</td>
<td>1.35±0.04*</td>
<td>1.38±0.11*</td>
<td>0.81±0.09</td>
<td>0.89±0.10</td>
</tr>
<tr>
<td>Nic+ dHβE</td>
<td>0.71±0.11*</td>
<td>1.90±0.15*</td>
<td>0.95±0.07</td>
<td>0.89±0.04</td>
<td>0.80±0.06</td>
<td>0.78±0.07</td>
</tr>
<tr>
<td>Nic+Mec</td>
<td>0.47±0.05*</td>
<td>2.40±0.17*</td>
<td>0.90±0.02</td>
<td>0.98±0.07</td>
<td>0.86±0.11</td>
<td>0.70±0.15*</td>
</tr>
</tbody>
</table>

*Note: Mec and dHβE, when applied alone, significantly down-regulated CRELD2 (8h) message both in wild type and ha4β2 SHEP1 cells (Figure 23B), but had no effect on rest of the gene transcripts indicated above.*significant at 0.05 level (2-tailed).
6.5. Influence of CRELD2 on αβ2 upregulation

Using yeast two-hybrid screening, Ortiz et al. (2005) found that the ER-localized human protein CRELD2 (Cysteine-rich EGF-like domain protein 2) interacts with both α4 and β2 nAChR subunits. Moreover, they showed that co-expression of CRELD2 with α4β2 in Xenopus oocytes markedly impairs functional expression of α4β2 nAChRs.

After noting the negative correlation between nicotine-induced α4β2 receptor up-regulation and CRELD2 expression, we measured surface receptor expression after silencing CRELD2 using siRNA designed to block the α and β isoforms (Maslen et al., 2006). qPCR indicated an 80% reduction in CRELD2 mRNA expression in cells transfected with 30nM CRELD2 siRNA (Fig.26). Transfection of hα4β2 SHEP1 cells with 30nM siRNA for 48h significantly increased basal α4β2 receptor expression compared to control cells (Fig.27A), while transfection with a scrambled negative-control siRNA had no effect. Further, application of 10µM nicotine to siCRELD2-transfected cells for 24h additively increased specific[^3]H] cytisine binding compared with nicotine-alone treatment (Fig.27A), while treatment with the scrambled negative-control siRNA had no effect on nicotine-induced up-regulation. These results suggest that CRELD2 negatively regulates α4β2 receptor expression. In addition we also found that incubation of either hα4β2 or wild-type SHEP1 cells with dHβE (100µM) or Mec (10µM) for either 8 or 24h decreases the expression of CRELD2 mRNA (Fig.27B).

*Figure 26. siCRELD2 silencing.*

Transfection with siRNA targeting CRELD2 for 48h achieved maximum silencing at 30nM siRNA, with no greater silencing at 100nM, as quantified by qPCR. Data represent mean ± SEM of three independent experiments. One-way ANOVA determined significant differences among the groups followed by a Tukey post-hoc test. **P<0.01.**
7. Discussion

We designed this study to determine whether α4β2 receptor up-regulation involves gene expression changes other than the receptor subunits genes themselves. α4β2 up-regulation is a post-transcriptional event, with no significant change in α4 or β2 mRNA (Marks et al., 1992), as we confirm here. In general, nicotine effects on gene expression in hα4β2 SH-EP1 cells are subtle to the point of being near the limit of detectable changes using qPCR. This is in keeping with the phenomenon of receptor up-regulation, which seldom involves more than a 2- to 3-fold increase in receptors.

Nicotinic receptor activation induces expression of immediate early genes such as c-fos and junB in systems with other nicotinic receptor subtypes (Greenberg et al., 1986; Pich et al., 1997), but not in our assays. We also did not
observe the same nicotine-induced gene changes reported in SH-SY5Y cells expressing α3 and α7 receptors. Dunckley and Lukas found that nicotine exposure alters the expression of contactin 1, UBE2C, UBE2S, and Parkin in SH-SY5Y cells (Dunckley and Lukas, 2003, 2006) with a heavy emphasis on the ubiquitin proteasomal degradation pathway. Kane et al. (Kane et al., 2004) also reported changes in the proteasomal-degradation pathway using microarray analysis of nicotine-treated rat brain, but the contributing receptor subtypes in this study are not clear.

**Human α4β2 SHEP1 cells**

Eaton et al. (2003) characterized hα4β2 receptors heterologously expressed in SHEP1 cells and suggested these cells as appropriate models for studies involving hα4β2 nAChRs. In later years, several studies validated hα4β2 cells as models for investigating functional properties of α4β2 nAChRs (Pacheco et al., 2001; Eaton et al., 2003; Wu, 2004). For example, Lukas and colleagues provided evidence for association between abnormal Aβ-amyloid deposition and α4β2 dysfunction in hα4β2 SHEP1 cells (Wu, 2004). Former students of our laboratory, Scott and Adham, used these cells as a model for investigating α4β2 nAChR upregulation. They found that 10µM nicotine subtly altered expression of 18 gene transcripts that are biologically related to inflammatory signaling. Similar effects were not observed in wild-type SHEP1 cells, lacking α4β2 or α7 nAChRs.

CREM and PAX5 were the only altered transcription factors identified by our microarray analysis. However, qPCR did not validate the PAX5 message changes. In contrast, CREM, isoforms of which can be negative modulators of CREB (Don and Stelzer, 2002), showed a significant but delayed induction at 24h, suggesting that CREM changes may occur too late to account for α4β2 receptor up-regulation. Nonetheless, recent evidence implicates that cAMP-dependent phosphorylation of α4β2 modulates expression of human α4β2 receptors (Pollock et al., 2009). This delayed increase in CREM mRNA in response to nicotine suggests a plausible negative feedback on α4β2-mediated signaling or up-regulation involving cAMP-CREB signaling, but this requires further investigation. CREM did show a positive correlation with the increased receptor up-regulation observed when antagonists were added to nicotine.

**ER resident proteins** Heteromeric α4β2 nAChRs exist in two stoichiometries: (α4)2(β2)3 and (α4)3(β2)2(34) and this stoichiometry may play a role in receptor up-regulation (Lopez-Hernandez et al., 2004). Folding, assembly, and glycosylation of these receptors occurs in the ER, and properly folded receptors are trafficked to the cell surface after passage through the Golgi (Sallette et al., 2005; Millar and Harkness, 2008). Nicotine readily crosses the cell membrane and may act as a pharmacological chaperone by interacting at the interface between α and β subunits,
thereby enhancing the stability and maturation of these receptors (Kuryatov et al., 2005; Sallette et al., 2005). In a different receptor family, recent work suggests that agonist occupancy of the receptor is necessary for AMPA receptor export from the ER (Coleman et al., 2009).

Several ER chaperones co-immunoprecipitate with nAChRs suggesting that more than one ER chaperone modulates nAChR maturation (Ortiz et al., 2005; Wanamaker and Green, 2007). Particularly, ER proteins BiP, CN, ERp57, UBXD4 and Ric-3 have been shown to regulate maturation and expression of nAChRs subtypes in Xenopus oocytes, neurons and mammalian cultured cells (Halevi et al., 2003; Lansdell et al., 2005; Wanamaker and Green, 2007; Rezvani et al., 2009). BiP plays an important role in the unfolded protein response to ER stress (Schroder and Kaufman, 2005), and although it appeared in the microarray results, qPCR indicated no significant change in BiP expression.

An eight hour exposure of hα4β2 SH-EP1 cells to nicotine enhanced expression of several possible ER chaperones (PDIA6, HERPUD1, and CRELD2) detected by the microarray, and qPCR confirmed these changes suggesting that these proteins might contribute to α4β2 up-regulation. qPCR did not confirm microarray predicted changes in PDIA4 mRNA. PDIA6, a different member of the PDI family, has two conserved thioredoxin domains (CXXC) that catalyze the formation, breakdown, and isomerization of disulfide bond between two cysteines (Kemmink et al., 1996; Freedman et al., 2002). HERPUD1 is an ER-localized protein and consists of an ubiquitin-like domain on the N-terminus, and the promoter region has c/EBP-ATF and ERSE sites that are regulated by both ER stress and cellular stress pathways (Kokame et al., 2000). HERPUD1 also promotes endoplasmic reticulum associated degradation (ERAD) by recruiting ubiquilins (Mishra et al., 2008) that negatively regulate up-regulation of neuronal nAChRs (Ficklin et al., 2005). However, treatment with α4β2 antagonists blocked nicotine-induced increases of HERPUD1 and PDIA6 transcripts while simultaneously enhancing receptor up-regulation, suggesting that any connection of these proteins to α4β2 receptor up-regulation may be indirect, but further work is required.

**CRELD2 mRNA depletion increases basal α4β2 receptors, but nicotine increases CRELD2 mRNA while inducing α4β2 upregulation**

CRELD2 interacts with both α4 and β2 subunits (Ortiz et al., 2005) and is an ER-resident protein with multiple splice variants (Maslen et al., 2006). Ortiz et al. (2005) found CRELD2 with a yeast-two-hybrid assay using the α4 cytoplasmic loop as bait and showed that CRELD2 overexpression decreases functional α4β2 expression in frog
oocytes. Our microarray data independently identified CRELD2 as a gene with nicotine-enhanced expression in hα4β2 SH-EP1 cells, and qPCR confirmed this at 8 and 24h. α4β2 antagonists Mec and dHβE decreased CRELD2 message and enhanced upregulation in hα4β2 SH-EP1 cells. Interestingly, these antagonists alone suppressed CRELD2 expression in wild-type SHEP1 cells lacking α4β2 or α7 receptors. This off-target effect suggests that antagonists work to suppress CRELD2 expression even in the absence of nicotinic receptors. We propose that this antagonist-induced decrease in CRELD2 mRNA levels may explain why antagonists elicit upregulation and synergistically potentiate nicotine-induced upregulation. Subsequently, siRNA knockdown of CRELD2 expression caused a significant increase in the basal expression of surface receptors. Microarray analysis and qPCR indicated that nicotine increases CRELD2 mRNA levels while simultaneously upregulating α4β2 receptors. In addition, nicotine exposure after depleting CRELD2 message additively increased nicotine-induced α4β2 upregulation. We hypothesize that CRELD2 might play a negative-feedback role in governing the extent of α4β2 upregulation, and nicotine-induced increases in CRELD2 message might limit the degree of α4β2 expression in chronic smokers. Presently, other members of our laboratory are investigating the following questions: 1. Do nicotine-induced increases in CRELD2 message translate to protein levels? 2. Does nicotine differentially regulate CRELD2 expression at times between 1h and 8h exposure? (Note: Nicotine exposure at 0.25 and 1h times had no effect on CRELD2 mRNA. However, an 8h exposure significantly increased CRELD2 mRNA). 3. Does CRELD2 silencing prevent Mec and dHβE enhancement of nicotine-induced upregulation? Our current hypothesis predicts that it should.

In summary, the first objective after the microarray analysis was to identify genes influencing α4β2 upregulation from a list of ~ 20 possible ER chaperones and cytosolic proteins. We provide early evidence that ER chaperones such as CRELD2 negatively regulate α4β2 surface receptor expression. Employing the procedure established here, Bharti Patel has screened (using correlation analysis) the following ER chaperones TMEM50B, FKBP11 and TMEM90, and found that mRNA levels of ER and Golgi membrane protein, TMEM50B, show strong positive association with surface α4β2 expression. Currently, siRNA-mediated silencing (screen 2) of TMEM50B in hα4β2 SHEP1 cells is in progress. Future work has to utilize the strategies outlined here to further identify genes contributing to α4β2 expression.
8. Future directions

The protein machinery involved in regulating the folding, assembly, maturation and trafficking of α4β2 receptors is far from being elucidated. We established a screening procedure to identify and test the function of genes influencing α4β2 receptor expression in hα4β2 SHEP1 cells. A preliminary screen by correlation analysis involving six gene transcripts identified three of six as possible candidates. Our working hypothesis was that because antagonists potentiate α4β2 upregulation, the expression of proteins influencing up-regulation must show strong correlation (positive or negative) with both nicotine-induced as well as antagonist-potentiated upregulation. An ER chaperone CRELD2 showed negative correlation with α4β2 expression and as anticipated siRNA-mediated silencing increased basal α4β2 expression. The remaining gene transcripts observed by microarray analysis can be screened using similar methods used here. Future studies:

1. Investigating whether α4β2 upregulation can be influenced by modulating the expression of genes identified by correlation analysis. Our preliminary data suggest that silencing CRELD2 increases the basal expression of α4β2 receptors compared with no siRNA treated controls in hα4β2 SHEP1 cells. Consistently, a non-viral over-expression of CRELD2 suppressed nicotine-induced α4β2 upregulation (unpublished data). siRNA-mediated silencing could be used to investigate the function of other gene transcripts identified by correlation analysis.

- Investigate the mechanistic role of cAMP-mediated signal transduction in α4β2 upregulation.
- Further investigate the role of ER Chaperones and other transmembrane proteins involved in assembly and maturation of α4β2 nAChRs.
- Try a combinatorial gene silencing. For instance, targeting both CRELD2 and CREM gene expression to find the sequence in which they affect α4β2 expression.
2. Investigating a combinatorial gene silencing (two) approach on α4β2 upregulation in human neuroblastoma cells. α4β2 nAChRs are pentamers and several proteins may participate to facilitate efficient folding, assembly, maturation and trafficking of receptors. It is very likely that one or more proteins function collectively to assist in surface receptor expression. Up to two different siRNAs targeting two different gene transcripts could be used to test if any of the genes work in tandem, and the sequence in which these genes influence α4β2 expression.

3. Investigating the mechanistic role of cAMP-dependent kinases on α4β2 upregulation. Research suggests that cAMP-dependent signal transducer PKA phosphorylates α4 and β2 subunits to influence receptor maturation and stability (Pollock et al., 2007; Pollock et al., 2009). Based on our preliminary data, mRNA expression of CREM, a transcription factor associated with cAMP-mediated signaling, showed a positive correlation with α4β2 upregulation. RNA interference can be used to specifically silence downstream effectors of cAMP-mediated signaling including PKA and CREM etc to test their apparent contribution to α4β2 upregulation. 10µM Forskolin and 30nM siCRELD2 can be used as positive controls to induce α4β2 upregulation.

4. Investigating the association between inflammatory responses and α4β2 expression. α4β2-mediated suppression of PIC production is described in chapter 2. While our study was in progress, Scott Roger’s laboratory has found that PICs IL-1β and TNFα modify neuronal nAChR assembly (Gahring et al., 2008). What has been suggested is that TNFα-induced p38 MAPK signaling might regulate the synthesis of ER chaperone like proteins, which could enhance the assembly of α4β2 nAChRs (Gahring et al., 2008). But little is known about the chaperones induced by p38 signaling and their impact on α4β2 receptor maturation. We identified several ER chaperones and cytosolic proteins during the microarray analysis. A good start to test the hypothesis for a reciprocal relation between inflammatory responses and nAChR expression will be to identify the ER chaperones from the available list (Table 1) that can be altered upon exposure to PICs. For example, cells can be stimulated with TNFα or IL-1β and the changes in message and protein levels for ER chaperones may be analyzed by qPCR and western blotting, respectively, compared with untreated controls.
Chapter 2

9. Problem statement. Neurological disorders such as AD, ischemic stroke, PD, epilepsy result in millions of deaths every year globally. Healthcare costs associated with these disorders are increasing exponentially (WHO: ‘global burden of neurological disorders’). Current drug treatment strategies are not very effective. Etiologies of neurological disorders could be environmental or genetic, but in most cases are not understood. However, post-mortem studies on human brains in patients with AD, PD and schizophrenia report severe inflammation (Ripoll et al., 2004), and the incidence of neurodegenerative diseases is significantly lower in chronic smokers (Fratiglioni and Wang, 2000; Picciotto and Zoli, 2008). Although nicotine is dangerous overall, it confers protection against neurological disorders (Kihara et al., 1998; Jonnala and Buccafusco, 2001; Hejmadi et al., 2003; Liu et al., 2007). The neuroprotective effects of nicotine could be due to one or more of the following reasons:

1. Similar to acetylcholine, nicotine mediates the release of other neurotransmitters such as dopamine, glutamate, GABA and serotonin. Thus, nicotine could be acting as a surrogate to acetylcholine in smokers.

2. Nicotine is immune-suppressive and anti-inflammatory (Kalra et al., 2004). Because neuroinflammation is a predominant feature of neurological disorders, continuous nicotine exposure in chronic smokers might be ameliorating CNS inflammation.

3. Nicotine induces cognitive and locomotor sensitization that could be beneficial for patients diagnosed with AD and PD, respectively (Newhouse et al., 2004; Singh et al., 2004).

Microglia are the first line of defense in the CNS and are under tight surveillance by neurons and astrocytes (Gonzalez-Scarano and Baltuch, 1999). Neuronal signaling regulates the inflammatory milieu of the CNS by keeping microglia quiescent or activating when necessary (Biber et al., 2007). While substantial evidence indicates vagus nerve activity regulates cytokine production in the periphery through activation of $\alpha 7$ nAChRs, their involvement in CNS cytokine regulation is still emerging. Recent studies have reported the expression of nAChR subtypes on microglia and astrocytes (Shytle et al., 2004), which emphasizes the diversity of cholinergic signaling in the CNS. $\alpha 4\beta 2$ nAChRs are major high-affinity binding sites for nicotine in the CNS. In addition, the concentration of nicotine achieved in a
smoker’s brain is about 50–300 nM, which is sufficient to activate α4β2 receptors (Wullner et al., 2008). Ryan et al. (2001) report the requirement of α4* nAChRs for nicotine-induced neuroprotection in 6-OHDA model for PD.

![Diagram](image)

**Figure 28. Neuroinflammation is a prominent feature of neurological disorders.**

Epidemiological data suggest negative correlation between tobacco smoking and development of neurodegenerative diseases. α4β2 are high-affinity receptors for nicotine in the CNS. We sought to investigate whether α4β2 nAChRs mediate protective effects of nicotine by ameliorating CNS inflammation in human neuroblastoma cells stably expressing α4β2 receptors.

In a preliminary microarray analysis, 10µM nicotine exposure suppressed pro-inflammatory cytokine (PICs) production in human neuroblastoma cultures stably expressing α4β2 nAChRs. In this study, therefore, we asked whether, similar to α7-mediated signaling, α4β2 receptor activation suppresses cytokine production, and if it does, then what are the signaling cascades downstream of receptor activation leading to cytokine regulation.

**10. Glossary**

**Immunology** - Refers to resistance to infections and diseases from foreign organisms. Immunity could be naturally acquired by a disease causing agent or artificially by vaccination. Based on the cell types involved immunity is further divided into humoral (secreted antibodies) and cell-mediated (T-lymphocytes). Immune cells originating from haematopoietic stem cells of bone marrow give rise to two progenitor cells: Myeloid (monocytes, macrophages, dendritic cells etc) and lymphoid.

**Lymphocytes** - Cells originating from lymphoid progenitors are called lymphocytes. Major lymphocytes are natural killer (NK), T- and B- cells. While B-cells mature into B-lymphocytes, T-cells (Fig.30) have to migrate to thymus.
gland to mature. Mature B-lymphocytes produce antibodies and T-lymphocytes differentiate into helper T- and cytotoxic T-cells.

**Antigen presenting cells (APCs)** - These are specialized leukocytes (Fig.31) that internalize the antigens and also express class II major histocompatibility (MHC) molecules on the cell surface that activate naïve T-cells (Aderem and Underhill, 1999). Ex. Dendritic cells, macrophages and B-lymphocytes.

**Microglia** - These cells are a type of glial cells that are often referred to as the resident macrophages of the CNS, and rightfully are the first line of defense against trauma, infections and ischemia (Gonzalez-Scarano and Baltuch, 1999). Evidence suggests that monocytes originating from bone marrow migrate to the CNS and differentiate into microglia. These cells constitute 10-15% of all cells in the CNS.

**IL 1β (interleukin 1-beta)** - This gene encodes a protein, which is a member of the interleukin 1 cytokine family. This pro-inflammatory cytokine is produced as a pro-protein and is cleaved by caspase 1 into an active form. IL-1β is an important mediator of inflammatory response. In addition, it is involved in a variety of cellular activities such as proliferation and apoptosis (Griffin and Mrak, 2002).

**IL-6 (interleukin 6 or interferon-beta 2)** - Like IL-1β, IL-6 is a pro-inflammatory cytokine secreted by activated macrophages and T-lymphocytes. In addition to pro-inflammatory properties, IL-6 displays anti-inflammatory
properties. IL-6 receptor (Fig.29) has glycoprotein (gp)130 as a common subunit, whose signal transduction involves activation of JAK-STAT and MAPK signaling pathway (Heinrich et al., 2003)

**TNFα (Tumor necrosis factor alpha)** - It is a pro-inflammatory cytokine that belongs to the tumor necrosis factor family. It is secreted by activated macrophages in response to stimulation by endotoxin LPS and is implicated in neuronal and peripheral diseases. Anti-TNFα blockers are very effective in treating several ailments, including rheumatoid arthritis and inflammatory bowel syndrome.

**NFκB (nuclear factor of kappa B)** - This gene encodes a 105kD protein that is cleaved into a 50 kD protein by the proteasome system. The 105 kD protein is a Rel protein-specific transcription inhibitor and the 50 kD protein is a DNA binding subunit of the NF-kappa-B (NFkB) protein complex. NFκB is activated post-transcriptionally by endotoxins, TNFα and other stimuli. Activated NFκB translocates to the nucleus to initiate transcription of genes involved in a variety of biological processes. It is a potential target for several inflammatory diseases and a variety of cancers (Chen et al., 1999).

**Alzheimer’s disease (AD)** - AD is a neurodegenerative disorder characterized by β-amyloid plaques and neurofibrillary tangles in the brain. The most common symptoms include loss of memory (dementia) and intellectual ability. Etiology of the disease is unknown, although reduced cholinergic activity and mutations in the amyloid precursor protein (APP) are hypothesized to cause AD (Hardy, 2004). Currently there is no cure for AD.

**Parkinson’s disease (PD)** - A progressive neurodegenerative disease resulting from loss of dopaminergic neurons in the nigro-striatal pathway. Research indicates accumulation of lewy bodies in post-mortem brains of patients with PD. In most cases PD is idiopathic (Hardy et al., 2003). Typical disease symptoms include tremor, rigidity and bradykinesia, though symptoms could be quite dissimilar among patients.

**IkBα** - An inhibitory protein, IkBα, forms a stable complex with NFκB in the cytoplasm and prevents its transcriptional activity until it is phosphorylated by inducers such as LPS or TNFα. Phosphorylated IkBα undergoes ubiquitination and breakdown, allowing NFκB to translocate to the nucleus (Chen et al., 1999).

**HMGB1** - This intracellular DNA binding protein is a late mediator of inflammation in sepsis (Ulloa et al., 2003). It is released by the activated macrophages and stimulates pro-inflammatory cytokine (IL-1β and IL-6) production. Specific inhibition of HMGB1 reverses lethality in murine models of sepsis (Wang et al., 2004).
11. **Background and significance**

11.1. CNS is ‘immune-privileged’ - Microglia are the first line of defense in the CNS and are under tight surveillance by the nervous system. A healthy blood brain barrier (BBB) prevents peripheral leukocyte infiltration, hence the term ‘immune privileged’, which, however, does not refer to lack of immunity. Substantial evidence indicates that leukocytes infiltrate the BBB during pathological conditions. In multiple sclerosis (MS), an elevated leukocyte count is found in the cerebrospinal fluid (CSF) (Engelhardt and Ransohoff, 2005). The leukocyte immigration is aided by the adhesion molecules and chemokine expression induced on BBB endothelium and choroid plexus epithelium during inflammation (Engelhardt and Ransohoff, 2005).

Microglia which constitute 10-15% of all cells in the CNS are key players in host cell defense mechanism (Gonzalez-Scarano and Baltuch, 1999). Similar to peripheral leukocytes, antigens stimulate microglial proliferation. Microglia migrate to the site of injury and express major histocompatibility class II molecules on the surface. Activated microglia release cytokines and chemokines to amplify inflammatory responses. Research indicates that neurodegenerative diseases are associated with excess secretory activity of microglia, which could eventually be neurotoxic (Gonzalez-Scarano and Baltuch, 1999).

Microglia play a dual role in amplifying the inflammatory signals and mediating neuronal damage. Research suggests that microglia can be protective or neurotoxic. For instance, experimental data suggest that lipopolysaccharide (LPS) induces a neurotoxic phenotype of microglia, whereas IL-4 induces a neuroprotective phenotype (Butovsky et al., 2005). Therefore, uncontrolled microglial proliferation could be conceived as overwhelmingly detrimental to the neurons, as evidenced in several animal models of neurodegeneration, including encephalomyelitis (EAE) (Shi et al., 2009).

**Neurons regulate microglia hypothesis** Neuronal signaling regulates the inflammatory milieu of the CNS by keeping microglia quiescent or activating when necessary (Fig.32). Under normal physiology, microglia are in a resting state and continuous neuronal surveillance is necessary to abrogate or antagonize cytokine activity (Biber et al., 2007). By contrast, neurons induce microglial activation during infections, trauma, ischemia etc. Additionally, for instance, peripheral nerve damage specifically activates the microglia corresponding to that neuron in the CNS, suggesting a precise regulation of microglial activity by neuronal signaling (Tracey, 2007).
11.2. ‘Off’ signal is as essential as ‘On’ signal

Two classes of signals regulate microglia. ‘Off’ signals are constitutive and restrict microglial activation, whereas ‘On’ signals initiate controlled microglia activation. The ‘On’ and ‘Off’ signals can be of two types: 1. Released, such as neurotransmitters, chemokines etc. 2. Membrane bound, such as the immunoglobulin super family, IgSF, molecules such as CD200 and CD47 (Engelhardt and Ransohoff, 2005). While the release site of these signals (type 1) is unknown, it is hypothesized to be related to synaptic activity (Biber et al., 2007).

Immune cells and inflammatory molecules such as cytokines and chemokines accumulate in neurodegenerative diseases such as AD, HD, stroke, PD and MS (Table 5). Accumulation of immune cells, including microglia, macrophages and T-lymphocytes harms neuronal cells in vivo and in vitro, although neurons do not express any known major histocompatibility markers that make them targetable to immune cells. Besides accumulation, a full blown immune invasion, though targeted against non-neuronal cells, can result in collateral damage to neurons. For instance, activated microglia damage neurons irrespective of the antigen specificity (Zipp and Aktas, 2006).

Figure 32. Neurons and astrocytes regulate the ‘On’ and ‘Off’ signals in the CNS.

Controlled activation illustrated on the left is beneficial and protects from harmful pathogens, whereas uncontrolled activation could cause severe neuronal damage (Takahashi et al., 2002; Biber et al., 2007).
Table 5. Accumulation of immune cells in the CNS contributes to inflammation-triggered neurodegeneration (Zipp and Aktas, 2006).

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<tr>
<th>Effectors</th>
<th>Disorder</th>
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<tr>
<td><strong>Cellular</strong></td>
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<tr>
<td>Macrophages and microglia</td>
<td>+</td>
<td>+</td>
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<tr>
<td>CD4+ T cells</td>
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<td>CD8+ T cells</td>
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<td>B cells</td>
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<tr>
<td>Neutrophils</td>
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<td><strong>Molecular</strong></td>
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<td>Glutamate</td>
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<td>Perforin</td>
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<tr>
<td>ROS, NO and ONOO</td>
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*Abbreviations: AD, Alzheimer’s disease; ALD, adrenoleukodystrophy; COX, cyclooxygenase; HAD, HIV-associated dementia; HD, Huntington’s disease; MS, multiple sclerosis; NO, nitric oxide; ONOO−, peroxynitrite; PD, Parkinson’s disease; ROS, reactive oxygen species; TRAIL, TNF-related apoptosis-inducing ligand; TNF, tumour necrosis factor. ‘+’ indicates there is evidence for involvement of a factor as an effector. Where a ‘+’ is shown in brackets, weak evidence hints to a possible involvement of the effector. ‘+−’ indicates that there is evidence that a factor is involved as an effector and, in parallel, the same factor also has opposite functions. Where no symbol is shown, there currently does not seem to be evidence either for or against an involvement.

11.3. Signaling pathways

In the CNS, nAChRs are localized to pre- and post-synaptic neuronal membranes. Acetylcholine released at the nerve terminals binds to and activates selective cationic channels allowing Na+, K+ and Ca2+ ions through the channel. Depolarization due to Na+ entry activates the voltage operated calcium channels (VOCC) leading to calcium influx. Ca2+ is then released from internal stores mediated by ryanodine and IP3 receptors (Fucile, 2004).

The increase in intracellular calcium can activate adenylyl cyclase (AC), protein kinase A (PKA), PKC, calcium calmodulin kinase (CaMK) and phosphatidylinositol-3-kinase (PI3K). Furthermore, these kinases phosphorylate downstream messengers such as ERK and MAPK leading to activation of transcription factors, including cAMP response element binding protein (CREB) (Dajas-Bailador and Wonnacott, 2004). However, recent studies suggest the involvement of two additional pathways JAK2-STAT3 and NFκB, which are critical for α7 nAChR-mediated anti-inflammation (Chen et al., 1999; Wang et al., 2004; de Jonge et al., 2005). Several factors including stress, UV and cytokines activate NFκB pathway and increase the production of PICs and chemokines. An inhibitory protein, IκBα, forms a stable complex with NFκB in the cytoplasm and prevents its transcriptional activity. Known inducers of the NFκB pathway LPS and TNFα activate NFκB translocation through IκBα phosphorylation.
Cholinergic stimulation via the vagus nerve has been suggested to antagonize immune cell cytokine production by inhibiting phosphorylation of IκBα and thereby preventing NFκB translocation to the nucleus (de Jonge and Ulloa, 2007; Tracey, 2007). Further, nicotine and other cholinergic agonists inhibit endotoxin LPS- and TNFα- induced NFκB activity by a similar mechanism (Yoshikawa et al., 2006; Dowling et al., 2007). However, signal mediators downstream of receptor activation are unknown.

Figure 33. Schematic of nAChR signaling.

A direct or an indirect calcium-dependent signaling has been suggested to regulate cAMP, PI3K and MAPK pathways. These signaling cascades are implicated in nicotine-induced addiction, cognitive benefits, mental alertness, neuroprotection and synaptic changes (Hejmadi et al., 2003; Dajas-Bailador and Wonnacott, 2004). Recent studies emphasize the role of two additional nAChR-mediated cascades: JAK-STAT and NFκB, which are hypothesized to play a critical role in regulation of vagus nerve dependent cytokine production. Note: As illustrated in the figure, the signaling cascades are not exclusive. Although separated by dotted boxes, there could be a cross-talk among these pathways. For instance, both cAMP-dependent PKA and STAT3 have been implicated to prevent NFκB translocation (Takahashi et al., 2002; Ancey et al., 2003; Heinrich et al., 2003; Arredondo et al., 2006)
JAK-STAT signaling

JAK-STAT signaling mediates important cellular processes such as apoptosis, cell proliferation and differentiation (O'Shea et al., 2002; Heinrich et al., 2003). For instance, IL-10R, IL-6R, INF-γR, growth hormone receptor (GH-R), prolactin receptor (Prl-R), IL-3R and IL-21 signal through the JAK-STAT pathway (Schindler et al., 2007) (Fig.34).

The human genome encodes four JAKs: JAK1, 2, 3 and TYK2 (Fig 35) (O'Shea et al., 2002). JAK1 and 2 are widely expressed and are associated with cytokine receptor signaling. While JAK3 associates with γC, Tyk2 is essential for LPS and INF-α/β signaling. These kinases with STATs form critical signal transducers for growth factor signaling as well. At least 7 mammalian STATs have been identified so far (STAT1-4, 5a, 5b and 6) (O'Shea et al., 2002; Heinrich et al., 2003). Phosphorylated JAKs interact with STATs encoding phosphotyrosine binding SH2

Figure 34. JAK-STAT signaling.

Cytokines bind to hetero- or homo-dimer receptors leading to activation of cytoplasmic JAK proteins. Activated JAKs phosphorylates tyrosine residues on cytokine receptors, providing sites for STAT binding. SH-2 domain of STAT determines the phospho-tyrosine binding. STATs are tyrosine phosphorylated by JAKs. Activated STATs form anti-parallel homo- or hetero-dimers and translocate to the nucleus. STATs bind to specific enhancer elements of DNA to promote gene transcription (O'Shea et al., 2002; Heinrich et al., 2003; Yamaoka et al., 2004). SOCS proteins negatively regulate IL-6R, IL-12R, IL-4R, GH-R, IL-4R, but not IL-10R
domain (Fig.36). Phosphorylated STATs form anti-parallel homo- or hetero-dimers and translocate to the nucleus to bind promoter elements of DNA to induce transcription of genes (Heinrich et al., 2003), including SOCS (suppression of cytokine synthesis) proteins (Yu et al., 2004) and Blc3. The SOCS proteins antagonize JAK-STAT signaling (Fig.34).

**Figure 35. Schematic of Janus kinases.**

*Abbreviations: FERM, 4.1/ezrin/radixin/moesin; SH2, src homology 2; JH, JAK homology domain; While FERM is involved in receptor interaction, both FERM and pseudokinase domains are implicated to regulate catalytic activity (O'Shea et al., 2002; Heinrich et al., 2003).*

**Figure 36. Schematic of signal transducers and activators of transcription (STAT).**

*Abbreviations: N-terminal domain; DBD, DNA binding domain; TAD, trans-activation domain; Y, tyrosine residue; S, serine residue (O'Shea et al., 2002).*

To date, SOCS-1, 2 and 3 are identified to negatively regulate INF-γR, IL-12, IL-4 and GH-R, but not IL-10R signaling (Schindler et al., 2007). SOCS proteins regulate JAK-STAT signaling by either binding to cytokine receptors and preventing JAKs phosphorylation or promoting ubiquitination of JAK-STAT complexes. In addition to SOCS proteins, tyrosine phosphatases such as CD45 and PTP1, and protein inhibitors of activated STAT (PIAS) proteins may modulate phosphorylation of JAKs and/or promote ubiquitination of phosphorylated-STAT dimers (Asadullah et al., 2003; Heinrich et al., 2003).

The following receptor types are involved in JAK2-STAT3 signaling:
i) **Cytokine receptors** – The domain interactions involved in JAK-STAT activation by cytokine and growth receptors are widely studied (Zhu et al., 1998; Haan et al., 2002; Heinrich et al., 2003).

Figure 37B shows the receptor homology of various cytokine receptors and species variants. Activation of erythropoietin receptor by its ligand erythropoietin (Epo) is discussed here. Epo binding recruits JAK2 to the cytoplasmic domain of its receptor. A conformational change results in activation of JAK2, which can phosphorylate other tyrosine residues of the receptor. Phosphorylated sites mediate STAT3 docking and phosphorylation. Activated STAT3 forms dimers and translocates to the nucleus. A highly conserved glycine residue in the cytokine receptors, indicated by an arrow in figure 37B, is critical for JAK2 activation. Pelletier et al. (2006) performed mutational analysis on erythropoietin receptor in DA3 cells in which $G^{277}$A and $G^{277}$C mutations significantly compromised the

![Diagram](image-url)
receptor function. Further, the following mutations completely abolished receptor function: G^{277}V, G^{277}P, G^{277}R, G^{277}Q and G^{277}E. In addition to the highly conserved glycine, the juxtamembrane (JM) motif (HRRTLQQKIW) and Box1 (PGIPSP) of domain 1 and Box2 (LEVL) of domain 2 are required for JAK2 binding to Epo receptors. The W^{258}A mutation (Fig.37A) in the JM motif significantly reduces the ability of Epo receptors to activate JAK2 (Pelletier et al., 2006).

ii) **GPCR (G-protein coupled receptors)** - Using mutational analysis in the cytoplasmic domains of a chemokine receptor CXCR4, Ahr et al. (2004) suggested that the intracellular loop 3A (Fig.38), is essential for JAK2 activation. Further, the tyrosine 157 located at the end of intracellular loop 2 (ICL2) is critical for recruiting STAT3. The sequence of events after chemokine ligand binding to its receptors is as follows: Chemokine binding induces a conformational change in ICL3 that enables JAK2 binding which then phosphorylates tyrosine 157 in the adjacent ICL2 resulting in STAT3 docking. It is noteworthy that ICL3 has no tyrosine residues, suggesting that

![Chemokine receptor CXCR4](image)

**Figure 38. Schematic of chemokine receptor CXCR4**

*The modified intracellular loop 3 (ICL3) is indicated as ΔICL3-A (SHSK), ΔICL3-B (KGHQ) and ΔICL3-C (QKRK). The transmembrane domains are indicated by gray bars. Red dotted circle show the Tyr-157 and the SHSK motif essential for JAK2-STAT3 activation. Modified from (Ahr et al., 2005).*

STAT3. The sequence of events after chemokine ligand binding to its receptors is as follows: Chemokine binding induces a conformational change in ICL3 that enables JAK2 binding which then phosphorylates tyrosine 157 in the adjacent ICL2 resulting in STAT3 docking. It is noteworthy that ICL3 has no tyrosine residues, suggesting that
tyrosine residues are not required for JAK2 activation. However, Ahr et al. (2005) found that SHSK motif (ΔICL3-A) in the N-terminal part of ICL3 is needed for JAK2 activation after chemokine ligand binding. Interestingly, the other tyrosine residues positioned at 65 in ICL2 and 76 and 135 in ICL1 were not involved in STAT3 binding. Further, it should be noted that the JAK2 docking motif is not conserved between the chemokine and cytokine receptors.

iii) α7 nAChR (Ligand-gated ion-channel receptor)- Using ELISA and immunoblotting, De Jonge et al. (2005) showed that nicotinic activation of α7 nAChRs attenuates cytokines TNFα and IL-6 production in peritoneal macrophages through JAK2-STAT3 signaling. Cholinergic agonists fail to attenuate cytokine production in cells expressing a mutated phosphorylation domain or DNA binding domain of STAT3 (de Jonge and Ulloa, 2007). However, the sites of JAK2 interaction and the tyrosine residues required for STAT3 docking are unknown.

11.4. Inflammatory reflex Peripheral immune response initiated by pathogens, injury or ischemia activates pro-inflammatory cytokine production (TNFα, IL-1β and IL-6) by macrophages (Tracey, 2007; Parrish et al., 2008).

Figure 39. Inflammatory reflex

Local injury or an infection stimulates controlled cytokine production, but overproduction could be harmful. Immune cell accumulation at the injury site relays sensory inputs to the nucleus tractus solitarius, which triggers the efferent vagus nerve to attenuate cytokine production to maintain homeostasis. α7 nAChRs-dependent signal transduction is critical in mediating the vagus nerve anti-inflammation (Tracey, 2002). Surgical removal or chemical block of the vagus nerve or specific α7 antagonists such as α-Bgt, d-TC or MLA block the inflammatory reflex (Tracey, 2007).
Neuronal activity regulates the balance between controlled and excessive cytokine production. Afferent vagus nerves relay immune cell responses from the site of injury to the brain, which subsequently stimulate the efferent vagus nerve activity to attenuate excess cytokine production. This efferent vagus nerve activity in response to peripheral injury is termed as the ‘inflammatory reflex’ (Fig 39).

**Convergence of neural inputs and cytokine production**- An exciting breakthrough that cholinergic activity regulates peripheral inflammatory responses has led to research into designing novel cholinergic therapeutics targeting the vagus nerve activity or receptors involved in mediating the vagus nerve signals. However, the release site of vagus nerve signals is still unknown. Tracey and colleagues investigated whether the vagus nerve stimulation, which regulates the activity of peripheral organs, including heart rate, could control cytokine production. Electrical stimulation of the vagus nerve attenuates cytokine production in wild-type mice, but not in α7 null mice, and also inhibits TNFα production during lethal endotoxemia in rats (Fig.40) (Borovikova et al., 2000; Wang et al., 2003).

![Figure 40. Cholinergic signaling cascade activated by nAChRs is still unclear.](image)

*Cholinergic activation blocks LPS- and TNFα-induced inflammation in macrophages expressing a message for α4, β2 and α7 nAChR subunits. Research suggests that inhibition of NFκB transactivation is a key step in attenuating cytokine production by immune cells, including macrophages and microglia. In addition, nicotine and other cholinergic agonists inhibit release of nuclear HMGB1 to improve survival in experimental model of sepsis (Tracey, 2007).*

The spleen is considered to be a major source of PICs during endotoxemia. Experimental evidence indicates that vagus stimulation suppresses cytokine production by macrophages in spleen and liver, which is lost in α7 KO mice or in presence of α7 antagonists such as α-Bgt, Mec or d-TC (Wang et al., 2003; de Jonge et al., 2005; Tracey, 2007).
Splenectomy fails to increase systemic pro-inflammatory cytokine release during endotoxemia, indicating a possible interface between the vagus nerve input and leukocyte (immune cell) production (Tracey, 2007).

11.5. Nicotine is immunosuppressive and anti-inflammatory-Tobacco smoke has nearly 4,500 chemicals that may be carcinogenic (Sopori, 2002; Tsurutani et al., 2005). Nicotine and tar are considered to be immunosuppressive; Kalra et al. (2004) suggest that animals exposed to nicotine remain immunosuppressed even after several weeks of exposure. They demonstrate that a nicotine patch inhibits antibody forming cell (AFC) response of spleen cells in animals immunized with sheep red blood cells. In addition, they showed that a nicotine patch suppresses humoral and cell-mediated immunity. In contrast, Ouyang et al. report that nicotine has little effect, whereas cigarette smoke extracts suppress human IL-1β, IL-2, INFγ and TNFα production (Ouyang et al., 2000).

Nicotine administration also inhibits endotoxin- and turpentine-induced inflammation (Kalra et al., 2004). For example, histopathological sections of turpentine-treated control mice had higher leukocyte accumulation, which was significantly attenuated by nicotine. Further, nicotine has been shown to inhibit influenza virus-induced leukocyte accumulation in lungs, implicating that nicotine might abolish migration of peripheral blood mononuclear cells (Razani-Boroujerdi et al., 2004; Saeed et al., 2005). Nicotine lowers infiltration and/or prevents the proliferation/expansion of immune cells. For instance, in a murine autoimmune encephalomyelitis (EAE) model of multiple sclerosis, 13mg/kg/day of nicotine treatment, significantly attenuated disease onset and severity compared with PBS treated control mice (Shi et al., 2009). The initial symptoms of the disease such as abnormal gait were evident 7 days after disease induction (caused by immunization of myelin oligodendrocyte glycoprotein, MOG, peptide), however, nicotine treatment at this point also reduced the severity of EAE. The pathological features of EAE include demyelination, axonal degeneration and cellular infiltration. Shi et al. (2009) showed that smoking relevant doses of nicotine relatively lowered infiltration of T-cells (CD3+, CD4+ and CD8+) and B-cells (CD3-CD19+) in the CNS, which were abundant in PBS-treated EAE mice.

Over the past decade numerous studies report expression of nAChRs on immune cells including microglia, macrophages and dendritic cells, and cholinergic activation suppresses cytokine production predominantly by two pathways: JAK-STAT and/or NFκB (see Table 6 below). Currently, a large number of cholinergic agonists are in pre-clinical and clinical trials to treat inflammatory conditions such as sepsis, hemorrhagic shock, ischemia, arthritis, ulcerative colitis and pancreatitis (Tracey, 2007).
Table 6. Summarized results of nAChR-mediated suppression of cytokine production by cholinergic agonists in different cell types.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>nAChR subunits</th>
<th>Stimulation</th>
<th>Cholinergic agonist</th>
<th>Cytokines/chemokines suppressed</th>
<th>Block</th>
<th>Mechanism</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human endothelial cells</td>
<td>α7,α5,α9</td>
<td>LPS (100ng/ml)</td>
<td>Nic, Capp55</td>
<td>TNFα</td>
<td>Mec, α-Bgt</td>
<td>↓ NFκB activation</td>
<td>(Saeed et al., 2005)</td>
</tr>
<tr>
<td>Human synovium/fibroblast like synoviocytes</td>
<td>α7; others subunits??</td>
<td>IL-1</td>
<td>Ach (1mM)</td>
<td>IL-6, CCL2, CCL3, CCL5</td>
<td>α7 siRNA, Mec, α-Bgt</td>
<td>JAK-STAT ????</td>
<td>(Waldburger et al., 2008)</td>
</tr>
<tr>
<td>RAW macrophages</td>
<td>α7; others subunits??</td>
<td>Endotoxin (6mg/kg)</td>
<td>Choline i.p.</td>
<td>TNFα</td>
<td>α7 KO</td>
<td>↓ NFκB, ↓ HMGB1</td>
<td>(Parrish et al., 2008)</td>
</tr>
<tr>
<td>Placental cells</td>
<td>α7; others subunits??</td>
<td>LPS (1-1000ng/ml)</td>
<td>Nic, GTS-21, Capp55</td>
<td>TNFα, IL-1β, IL-6</td>
<td>--------</td>
<td>↓ NFκB activation</td>
<td>(Dowling et al., 2007)</td>
</tr>
<tr>
<td>Human monocytes (PBMC)</td>
<td>α7; others subunits??</td>
<td>LPS 1μg/ml</td>
<td>Nic (0.1-100μM)</td>
<td>IL-18, IL-12</td>
<td>Mec, α-Bgt</td>
<td>↑ PGE2 synthesis</td>
<td>(Takahashi et al., 2006)</td>
</tr>
<tr>
<td>Human macrophages</td>
<td>α7; others subunits??</td>
<td>LPS</td>
<td>Nic (1-10μM)</td>
<td>serum HMGB1</td>
<td>α7 antisense, α-conotoxin, Mec</td>
<td>↓ NFκB</td>
<td>(Wang et al., 2004)</td>
</tr>
<tr>
<td>Human monocytes (PBMC)</td>
<td>α7; others subunits??</td>
<td>LPS 1μg/ml</td>
<td>Nic</td>
<td>TNFα, MIP1α, MIP-1β</td>
<td>α-Bgt</td>
<td>↓ IkBα phosphorylation, ↓NFκB activation</td>
<td>(Yoshikawa et al., 2006)</td>
</tr>
<tr>
<td>Peritoneal Macrophages</td>
<td>α7; α4β2</td>
<td>LPS 100ng/ml</td>
<td>Nic (1-1000nM)</td>
<td>TNFα, MIP2, IL-6</td>
<td>Curare, α-Bgt, Mec, Hexamethonium</td>
<td>JAK2-STAT3 activation</td>
<td>(de Jonge et al., 2005), Van der Zanden et al., 2009</td>
</tr>
<tr>
<td>Oral keratinocytes</td>
<td>α7; others subunits??</td>
<td>Exposed to ADDS or Nic</td>
<td>κB</td>
<td>α7 siRNA, α-Bgt</td>
<td>JAK2-STAT3 activation</td>
<td></td>
<td>(Arredondo et al., 2006)</td>
</tr>
<tr>
<td>Microglial cells (Mouse cortex)</td>
<td>α7; others subunits??</td>
<td>LPS 100ng/ml</td>
<td>Ach, Nic</td>
<td>TNFα</td>
<td>Mec, α-Bgt</td>
<td>↓ p44/42, p38 phosphorylation</td>
<td>(Shytle et al., 2004)</td>
</tr>
<tr>
<td>Alveolar macrophages</td>
<td>α4 and β2 subunits</td>
<td>Legionella pneumophila</td>
<td>Nicotine (0.1-10ug/ml), DMPP</td>
<td>IL-6, IL-12, TNFα</td>
<td>d-TC</td>
<td>---------------------------</td>
<td>(Matsunaga et al., 2001)</td>
</tr>
<tr>
<td>Mice skin</td>
<td>α4,β2,α7, α3, β4</td>
<td>UV(280-320nM)</td>
<td>Nic</td>
<td>IL-1β, IL-6, TNFα</td>
<td>α7 KO</td>
<td>↑ SOCS3</td>
<td>(Osborne-Hereford et al., 2008)</td>
</tr>
</tbody>
</table>
12. Objectives and specific aims

-To investigate the signaling pathways of α4β2 leading to suppression of inflammatory and immune responses-

There is no cure for neurological disorders but substantial evidence suggests that smokers are less susceptible to AD and PD, and schizophrenic smokers self-medicate with nicotine and report marked improvement in mental alertness and cognition (Cattapan-Ludewig et al., 2005). There is a significant loss of α4β2 nAChRs in patients with neurological disorders, although the loss may not always correlate with the disease severity (Court et al., 2001; Perry et al., 2001; Kas et al., 2009). Nicotine protects from neuronal insults in experimental models of MS (Shi et al., 2009), PD and AD (Picciotto and Zoli, 2008), which is lost in α4* knockout animals (Ryan et al., 2001; Picciotto and Zoli, 2008). However, the molecular mechanisms underlying α4* nAChR-mediated neuroprotection are still unclear. Over the past decade several studies report α7-mediated anti-inflammatory effects in the periphery (Wang et al., 2003; Saeed et al., 2005; Tracey, 2007; Waldburger et al., 2008). Microarray analysis (Scott & Adham) showed that nicotine suppresses pro-inflammatory cytokine expression in SHER1 cells expressing hα4β2 receptors. However, SHER1 cells do not express α7 nAChRs unless co-transfected with Ric3 (Sweileh et al., 2000; Lee et al., 2009). The objective was to examine whether α4β2 regulate immune responses and also evaluate the involvement of calcium, and cAMP- and JAK2-STAT3 signaling pathways in suppressing endotoxin-induced inflammation. The specific aims of this study were:

1. To validate the microarray analysis of nicotine-induced cytokine suppression using qRT-PCR- In a preliminary microarray study, we found that 10μM nicotine altered expression for approximately 18 gene transcripts related to inflammatory pathways in human α4β2 SHER1 cells. As a starting point, we selected PICs IL-1β and IL-6 to perform further investigation. First, we validated gene suppression of cytokines and chemkines using real-time PCR; GAPDH served as an internal control for qPCR. Nicotine induced gene alterations were subtle, and further, gene alterations may not reflect protein changes and so we measured protein levels of PICs by ELISA. We hypothesized that nicotine-induced changes in PIC expression were α4β2-receptor mediated. To test this, we used specific antagonists dHβE (competitive) or Mec (channel blocker) and co-incubated with nicotine before quantifying the message and proteins levels of IL-1β and IL-6. Wild-type SHER1 cells lacking α4β2 served as negative controls.
2. To examine whether α4β2 nAChR-mediated suppression of cytokines is through the NFκB pathway.

Previous studies suggest that cholinergic agonists inhibit PIC production in macrophages expressing messages for α4 and β2 subunits (Tracey, 2007). To test whether nicotine exposure inhibits constitutive NFκB activity, we transfected hα4β2 cells with NFκB-promoter containing luciferase vector and incubated them with varying concentrations of nicotine, with or without specific antagonists dHβE or Mec. We also investigated whether α4β2-receptor signaling interferes with endotoxin-induced NFκB transactivation. To test this, NFκB-reporter transfected cells were stimulated with varying concentrations of bacterial endotoxin LPS with or without nicotine pre-treatment and determined the NFκB transactivation and PIC production. The results obtained were compared with NFκB transfected naïve (unstimulated) hα4β2 SHEP1 cells. Further, wild-type SHEP1 cells that do not express any known nAChRs were used as a negative control.

3. To investigate the mechanistic role of cAMP-PKA and JAK2-STAT3 signaling cascades in α4β2-mediated regulation of cytokine production. First, we tested the involvement of calcium using a cell permeable calcium chelator BAPTA-AM on NFκB translocation (luciferase assay) and IκBα-phosphorylation (immunoblot and ELISA). Our preliminary results indicate that nicotine elevates cAMP levels in hα4β2 SHEP1 cells, with 4h exposure causing a maximum accumulation. Ouchi et al. (2000) found that activators and stimulators of PKA including forskolin and cAMP analogs inhibit transactivation of NFκB in other systems. To test the hypothesis that increases in calcium and/or accumulation of cAMP and its dependent PKA are involved in suppression of NFκB, we examined the effects of nicotine on LPS-induced NFκB translocation in the presence or absence of a specific PKA inhibitor peptide (PKI 14-22 amide). The adenylate cyclase stimulant forskolin was used as a positive control.

The JAK2-STAT3 signaling is involved in α7-mediated anti-inflammation (de Jonge et al., 2005; Arredondo et al., 2006). Hence, we tested the involvement of JAK2-STAT3 signaling using a broad spectrum JAK inhibitor I, a specific JAK2 inhibitor AG-490 and a specific STAT3 inhibitor NSC74859 on LPS-induced NFκB translocation and an upstream IκBα-phosphorylation.
**Aim #1** Investigate the role of α4β2 nAChRs in mediating anti-inflammatory effects using qPCR and ELISA.

**Aim #3** Which signaling cascades are involved in α4β2-mediated anti-inflammatory effects?

**Aim #2** Does NFκB pathway mediate nicotine-induced suppression of cytokine production?

**Pre-inflammatory cytokines IL-1β, IL-6**
13. Material and methods

**Cell culture and Reagents** - SHEP1 human α4β2 cells were a generous gift from Ron Lukas. Nicotine and endotoxin lipopolysaccharide were purchased from Sigma (St Louis, MO, USA). NFκB peptide inhibitor SN50, JAK inhibitor I, JAK2 inhibitor AG-490, calcium chelator BAPTA-AM, forskolin, STAT3 inhibitor VI NSC74859, PKA inhibitor peptide PKI 14-22 amide and calcium ionophore A23187 were obtained from calbiochem (San Diego, CA, USA). Primary antibodies were purchased from Cell signaling technology (Danvers, MA, USA) and secondary antibody was from Millipore (MA, USA).

**AG-490** - It is a cell permeable and specific inhibitor of JAK2. It is synthesized on the basic structure of erbstatin (Miyamoto et al., 2001). Meydan et al. (1996) indicated that AG-490 has anti-JAK2 activity and inhibits the growth of a human B-precursor leukemic cell line. Miyamoto et al. (2001) showed that 25µM AG-490 significantly prevented JAK2 phosphorylation in three different cell lines. In addition, they demonstrated that AG-490 did not inhibit other members of JAK family.

**NSC74859** - It is a cell permeable and potent inhibitor of STAT3 phosphorylation. It binds to STAT3 SH2 domain and prevents STAT3 activation, and thereby inhibiting dimerization, DNA-binding and gene transcription. Lin et al. (2009) assessed the STAT3 inhibition by NSC74859 in 6 different cell lines and suggested that the IC50s vary from 15µM to 250µM depending on the cell type.

**Cytokine ELISA** - To determine α4β2-mediated suppression of IL-1β and IL-6, cells seeded onto 6-well dishes were treated with nicotine, or nicotine plus dHβE or Mec for 48hrs. The experiment was performed according to the manufacturer’s protocol (eBioscience). To normalize for cell numbers, cells were lysed and the protein concentration (OD₂₈₀) was determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer. Normalized cell lysate was added onto primary antibody-coated plates and allowed to incubate overnight at 4°C. The following day, plates were incubated with biotin-conjugated antibody at room temperature for 1h, and avidin-HRP was added to the cells and allowed to bind for 30 min. Following incubation, TMB substrate was added and the reaction was blocked after 15 min. The absorbance was measured using a BioTek-HT UV-Vis microplate reader at 450 nm.
**Phospho-IκBα ELISA** - Phospho-IκBα levels were examined in hα4β2 SHEP1 cells by FunctionELISA IκBα assay kit (Active motif, CA, USA). Cells plated in 6-well plates and grown for 2 days were pre-treated with nicotine, BAPTA-AM or AG-490 before stimulation with or without 1µg/ml LPS for 25 min. Subsequently, cells were washed 2x with ice-cold PBS and a cell scraper was used to remove cells. Cells were centrifuged for 10min at 1000rpm at 4°C and then pellets were incubated in lysis buffer containing protease inhibitor for 30 min on ice. Samples were again centrifuged for 20 min at 14,000 x g at 4°C. Samples were stored at -80°C until further use. Protein concentration was determined by a NanoDrop® ND-1000. 100µg of cell lysate was added to capture antibody pre-coated plates and incubated for 4h at 4°C. Plates were thoroughly washed and detection antibody was added and allowed to incubate for 1h at RT. After a second wash, secondary antibody was added for 1h before quantifying the signal using a chemiluminescent substrate.

**Calcium indicator assay** - Fluo-4 Direct calcium assay kit (Invitrogen, CA, USA) was used to measure nicotine- and A23187-induced increase in intracellular calcium. Cells plated in a 96-well plate were grown for 24h before incubating for 45min with Fluo-4 reagent solution at 37°C, and then exposed to BAPTA-AM for 30 min. Fluorescence was measured using a BioTek-HT microplate reader at excitation, 485±20 nm and emission, 520±20 nm. Calcium ionophore A23187 and nicotine were added onto cells by an automated dispenser and fluorescence measurements were read at 3 sec intervals for 1 min. All the readings were monitored within 4h of adding the Fluo-4 reagent solution without a medium change. Baseline measurements were recorded after dispensing either PBS or methanol (carrier for A23187).

**cAMP ELISA** - Cells grown to confluence in 6-well dishes were incubated with 10µM nicotine or 10µM forskolin and intracellular cAMP levels were monitored using competitive ELISA kit (R & D systems, Minneapolis, MN). Manufacturer’s recommended procedure was followed. The activity was measured as absorbance at 450 nm on a BioTek-HT microplate reader.

**NFκB secreted luciferase reporter assay** - SH-EP1 hα4β2 cells were grown to confluence in a 24 well plate and transfected with pNFκB luciferase vector (Clontech), which contains the NFκB promoter element upstream of a Metridia luciferase reporter gene. Following 10h of transfection and a media change, cells were treated with or without nicotine or nicotine plus varying concentrations of Mec or dHBE for 4h. 50µl of sample media was assayed immediately for secreted luciferase activity using a BioTek-HT microplate reader.
Western blotting- To investigate whether α4β2-mediated anti-inflammatory effect involves phosphorylation of IκBα, we probed using antibodies against total IκB and phospho-IκBα (phosphorylated IκBα undergoes ubiquitination and allows translocation of NFκB to the nucleus) after treating hα4β2 SHEP1 cells with nicotine/BAPTA-AM/ AG490 inhibitor/LPS. Briefly, cells seeded onto 6-well dishes were treated with the varying concentrations of nicotine with or without inhibitors. Following incubation, cells were washed 3X with PBS and lysed using Mammalian protein extraction reagent (Pierce). Protein concentration was determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer. Equal concentrations of protein was heated at 95°C for 5min before loaded onto pre-cast gels (Pierce). SDS-gel was run at ~140V for 45min at RT. Gel was transferred to a PVDF membrane at 30mV for 2h at 4°C. Membrane was then stained with panceau-S reagent to visualize efficiency of protein transfer before incubating with blocking buffer (1X TBS, 5% w/v nonfat dry milk, 0.1% tween) at RT for 1hr. The blot was incubated in primary antibody buffer (0.2g/ml of antibody and blocking buffer) overnight at 4°C. A thorough wash (5X with TBS/T) was followed by incubation with secondary antibody (HRP-conjugate) buffer (10ng/ml ) at RT for 1hr. The washing step was repeated before a final exposure to a chemiluminescent substrate (SuperSignal West Pico, Thermo Scientific).

14. Results

14.1. Nicotine suppresses cytokine production While investigating the gene alterations contributing to α4β2 upregulation we discovered that 10μM nicotine significantly altered gene expression for 18 probe sets on Affymetrix HG-U133A arrays that belong to inflammation or immune response pathways (Table 9). We selected the following gene transcripts for validation by qPCR: Interleukins IL-1β, IL-11 and IL-6, chemokine CXCL2 and SOD2. As predicted by the microarray, nicotine significantly increased IL-11 expression (8 and 24h) but decreased the expression of the chemokine CXCL2 (1, 8 and 24h) and the PICs IL-1β and IL-6 at both 8h and 24h when normalized with GAPDH expression and compared with untreated controls (Table 7). In addition, we also analyzed the gene transcripts NFκB and TNFα, and show that 10μM nicotine does not alter the message for either gene, which is consistent with previous literature regarding α7 activation (de Jonge et al., 2005).
Table 7. Inflammation related gene transcripts selected for validation by qPCR.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Accession #</th>
<th>mRNA fold change by qPCR (10µM Nicotine exposure)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1h</td>
</tr>
<tr>
<td>IL-1β</td>
<td>NM_000576</td>
<td>1.03±0.02</td>
</tr>
<tr>
<td>IL-6</td>
<td>NM_000600</td>
<td>0.80±0.02</td>
</tr>
<tr>
<td>CXCL2</td>
<td>NM_002089</td>
<td>0.45±0.02*</td>
</tr>
<tr>
<td>SOD2</td>
<td>NM_000636</td>
<td>0.73±0.01*</td>
</tr>
<tr>
<td>IL-8</td>
<td>NM_000584</td>
<td>1.00±0.04</td>
</tr>
<tr>
<td>IL-11</td>
<td>NM_000641</td>
<td>0.82±0.18</td>
</tr>
<tr>
<td>TNFα</td>
<td>NM_000594</td>
<td>1.06±0.21</td>
</tr>
<tr>
<td>NFKB1(NFκB)</td>
<td>NM_003998</td>
<td>1.10±0.01</td>
</tr>
</tbody>
</table>

* Gene transcripts predicted by microarray but not validated by qPCR. The primers used in qPCR are shown in Table 8 below.

* Significant at 0.05 level (n=3 with quadruplicate readings).

Table 8. qPCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5’ - TTC GAC AGT CAC CGC CAT CTT CTT - 3’</td>
<td>5’ - ACC AAA TCC GTT GAC TCC GAC CTT - 3’</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5’ - AGA GTG CTG TGC TGA ATG TGG ACT - 3’</td>
<td>5’ - TGG GCA TTG GTG TAG ACA ACA GGA - 3’</td>
</tr>
<tr>
<td>IL-6</td>
<td>5’ - AAA TTC GGT ACA TCC TCG ACG GCA - 3’</td>
<td>5’ - GTG CCT CTT TGC TGC TTT CAC ACA - 3’</td>
</tr>
<tr>
<td>TNF</td>
<td>5’ - AGG ACG AAC ATC CAA CCT TCC CAA - 3’</td>
<td>5’ - TTT GAG CCA GAA GAT GGT GAG GGT -3’</td>
</tr>
<tr>
<td>NFκB</td>
<td>5’ - TGT AAC TGC TGG ACC CAA GGA CAT - 3’</td>
<td>5’ - AAA GCT GTA AAC ATG AGC GGC ACC - 3’</td>
</tr>
<tr>
<td>SOD2</td>
<td>5’ - AGTTGCTGGAAGCACCATACAAACGTG - 3’</td>
<td>5’ - TAAAGCCTGTGTGTTCCCTTTGAGTG - 3’</td>
</tr>
<tr>
<td>IL-8</td>
<td>5’ - ACA AGT CCT TGT TCC ACT GTG CCT - 3’</td>
<td>5’ - TCA CTG TGA GGT AGT GTG GCT - 3’</td>
</tr>
<tr>
<td>CXCL2</td>
<td>5’ - CCA AAC CGA AGT CAT AGC CAC ACT - 3’</td>
<td>5’ - AAC ACA TTA GGC GCA ATC CAG GTG - 3’</td>
</tr>
<tr>
<td>TLR4</td>
<td>5’ - TCC TGC AAT GGA TCA AGG ACC AGA - 3’</td>
<td>5’ - GCC AGC AAG AAG CAT CAG GTG AAA -3’</td>
</tr>
</tbody>
</table>

63
Table 9. Inflammation and immune response genes

Inflammation and immune response gene transcripts altered by 10µM nicotine in hα4β2 SHEP1 cells in microarrays compared with untreated controls (Categorized by Pathway Architect™ (Stratagene)).

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene ID</th>
<th>Gene title</th>
<th>Accession #</th>
<th>Time point</th>
<th>Fold Change (p&lt;0.005)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAMTS1</td>
<td>9510</td>
<td>ADAM metallopeptidase with thrombospondin type 1 motif, 1</td>
<td>NM_006988</td>
<td>8h</td>
<td>0.782</td>
</tr>
<tr>
<td>CCL20</td>
<td>6364</td>
<td>Chemokine (C-C motif) ligand 20</td>
<td>NM_004591</td>
<td>8h</td>
<td>0.086</td>
</tr>
<tr>
<td>CXCL1</td>
<td>2919</td>
<td>Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)</td>
<td>NM_001511</td>
<td>8h</td>
<td>0.444</td>
</tr>
<tr>
<td>CXCL2</td>
<td>2920</td>
<td>Chemokine (C-X-C motif) ligand 2</td>
<td>NM_002089</td>
<td>8h</td>
<td>0.223</td>
</tr>
<tr>
<td>CXCL3</td>
<td>2921</td>
<td>Chemokine (C-X-C motif) ligand 3</td>
<td>NM_002090</td>
<td>8h</td>
<td>0.134</td>
</tr>
<tr>
<td>CD55</td>
<td>1604</td>
<td>CD55 molecule, decay accelerating factor for complement</td>
<td>NM_000574</td>
<td>8h</td>
<td>0.429</td>
</tr>
<tr>
<td>IL11</td>
<td>3589</td>
<td>Interleukin 11</td>
<td>NM_000641</td>
<td>8h</td>
<td>0.392</td>
</tr>
<tr>
<td>IL1β</td>
<td>3553</td>
<td>Interleukin 1, beta</td>
<td>NM_000576</td>
<td>8h</td>
<td>0.346</td>
</tr>
<tr>
<td>IL6</td>
<td>3569</td>
<td>Interleukin 6</td>
<td>NM_000600</td>
<td>8h</td>
<td>0.241</td>
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<tr>
<td>IL8</td>
<td>3576</td>
<td>Interleukin 8</td>
<td>NM_000584</td>
<td>8h</td>
<td>0.262</td>
</tr>
<tr>
<td>INHBA</td>
<td>3624</td>
<td>Inhibin, beta A</td>
<td>NM_002192</td>
<td>8h</td>
<td>0.267</td>
</tr>
<tr>
<td>PTGS2</td>
<td>5743</td>
<td>Prostaglandin-endoperoxide synthase 2</td>
<td>NM_000963</td>
<td>8h</td>
<td>0.26</td>
</tr>
<tr>
<td>SOD2</td>
<td>6648</td>
<td>Superoxide dismutase 2, mitochondrial</td>
<td>NM_000636</td>
<td>8h</td>
<td>0.478</td>
</tr>
<tr>
<td>TNFAIP3</td>
<td>7128</td>
<td>Tumor necrosis factor, alpha-induced protein 3</td>
<td>NM_006290</td>
<td>8h</td>
<td>0.316</td>
</tr>
<tr>
<td>TNFRSF6</td>
<td>355</td>
<td>Tumor necrosis factor receptor</td>
<td>NM_032945</td>
<td>8h</td>
<td>0.608</td>
</tr>
<tr>
<td>HSP90B1</td>
<td>7184</td>
<td>Heat shock protein 90kDa beta (Grp94), member 1</td>
<td>NM_003299</td>
<td>8h</td>
<td>1.298</td>
</tr>
<tr>
<td>VEGFc</td>
<td>7424</td>
<td>Vascular endothelial growth factor C</td>
<td>NM_005429</td>
<td>8h</td>
<td>0.804</td>
</tr>
</tbody>
</table>

14.2. Antagonists block nicotine-induced suppression of cytokines- Preliminary microarray analysis indicated that continuous exposure to 10µM nicotine suppresses pro-inflammatory cytokines (PICs), chemokines and other immune response gene transcripts. We limited further evaluation to nicotine-induced suppression of the PICs IL-1β and IL-6 as examples of these effects. After qPCR validation of nicotine-induced PIC suppression (Table 7), we evaluated whether
this effect was α4β2-receptor mediated. Co-incubation of 10μM nicotine with a specific antagonist dHβE (100μM) or a non-specific channel blocker Mec (10μM), countered nicotinic attenuation of PICs mRNA levels (Table 10). The gene alterations were subtle to the point of being detected by qPCR and showed less than a 2-fold change between control and nicotine treated samples. Therefore, we analyzed IL-1β and IL-6 protein levels in hα4β2 SHEP1 cells and find that nicotine attenuates PIC proteins in a dose-dependent manner, with 10μM causing a 50 % reduction (Fig 41A). By contrast, addition of 100μM dHβE or 10μM Mec at the time of nicotine treatment for 48h reverses nicotine-induced suppression of IL-1β and IL-6 protein levels, as measured by ELISA (Fig 41B). Application of antagonists alone had no effect on either IL-1β or IL-6 message or protein levels (data not shown). The antagonist inhibition suggests that nicotinic suppression of PICs requires α4β2 receptor activation. Further, similar effects were not observed in wild-type SHEP1 cells lacking α4β2 receptors.

### Table 10. Effect of antagonists dHβE and Mec on nicotine-induced gene alterations.

<table>
<thead>
<tr>
<th></th>
<th>IL-1β(8h)</th>
<th>IL-6(8h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.03±0.02</td>
<td>1.08±0.02</td>
</tr>
<tr>
<td>Nicotine</td>
<td>0.77±0.03*</td>
<td>0.70±0.06*</td>
</tr>
<tr>
<td>Nic+ dHβE</td>
<td>0.88±0.18</td>
<td>0.93±0.11</td>
</tr>
<tr>
<td>Nic+Mec</td>
<td>1.16±0.20</td>
<td>0.99±0.17</td>
</tr>
</tbody>
</table>

Note: Mec and dHβE, when applied alone both in wild type and hα4β2 SHEP1 cells had no effect on IL-1β and IL-6 gene transcripts. *Significant at 0.05 level (2-tailed).

14.3. Nicotine inhibits basal NFκB transactivation in hα4β2 SHEP1 cells - We performed qPCR analysis on hα4β2 SHEP1 cells after 1, 8 and 24h exposure to 10μM nicotine and found that nicotine had no effect on mRNA levels of NFκB (Table 7). Translocation of the transcription factor NFκB from the cytoplasm to the nucleus is an important canonical step in the expression of PIC’s such as IL-1β and IL-6 (Perkins, 2007). To examine whether nicotine prevents NFκB translocation, we transfected hα4β2 SHEP1 cells with NFκB luciferase vector, and found that nicotine dose-dependently inhibited NFκB activity compared with untreated controls; a 4h treatment with 3μM nicotine gave a maximum effect of approximately a 65% decrease in relative luciferase activity, with 1μM blocking approximately 55% (Fig. 42A).
Antagonists block nicotine-induced suppression of basal IL-1β and IL-6 expression.

*a4b2 SHEP1* cells seeded onto 6 well dishes were exposed to nicotine or nicotine plus 100µM dHβE or 10µM Mec. 

(A) Protein levels of IL-1β and IL-6 were estimated after 48h exposure to varying concentrations of nicotine, as determined by ELISA. (B) Protein levels of IL-1β (light bars) and IL-6 (dark bars) significantly dropped after 48h exposure to 1µM nicotine but not when treated with 1µM nicotine plus 100µM dHβE or 10µM Mec. Data represent mean ± SD of three independent experiments for part A, four experiments for part B. IL-1β (*p<0.05, **p<0.01); IL-6 (#p<0.05, ##p<0.01).

Nicotine inhibits translocation of basal NFκB.

A) Human α4β2 SHEP1 cells were grown to confluence and transfected with pNFκB-MetLuc2-Reporter vector for 10h and then incubated with varying concentrations of nicotine for 4h. Sample supernatant was collected to determine luciferase activity. B) After 10h of reporter vector transfection, cells were co-incubated with control media, or 1µM nicotine with or without indicated concentrations of Mec or dHβE before measuring luciferase activity. Data represent mean±SEM of three independent experiments with triplicate samples. ANOVA determined the statistical difference among different groups. *p<0.05; #p< 0.05, significantly different from 1µM nicotine treatment.
Next, to determine whether this effect was receptor dependent, cells were co-incubated with indicated doses of antagonists Mec and dHβE. Both antagonists blocked nicotine-induced suppression of NFκB translocation (Fig 42B), with 5μM Mec and 50μM dHβE completely blocking the effects of 1μM nicotine applied for 4h. Thus, nicotinic attenuation of PICs may be mediated through NFκB pathway, and nicotine inhibits NFκB translocation post-transcriptionally without affecting the mRNA levels of NFκB, which is consistent with previous literature.

14.4. Nicotine blocks LPS-induced NFκB pathway- Nicotinic activation of α7 receptors attenuates endotoxin-induced NFκB translocation and pro-inflammatory cytokine (TNFα, IL-1β and IL-6) production (Borovikova et al., 2000; Wang et al., 2003; Wang et al., 2004). To investigate whether α4β2 nAChRs mediate anti-inflammatory effects similar to α7 activation, we stimulated hα4β2 SHEP1 cells with varying concentrations of the bacterial endotoxin LPS. SHEP1 cells express a message for the LPS receptor, Toll-like receptor 4 (TLR4), as determined by qPCR and DNA sequencing (not shown). Initial experiments determined the concentration of LPS to induce NFκB translocation (Fig. 43A). 1µg/mL and 3µg/mL LPS caused a ~2 fold and ~ 4-fold increase, respectively, in NFκB activity. However, pre-treatment with nicotine effectively attenuated LPS-induced NFκB transactivation in a dose-dependent manner, as measured by NFκB-reporter assay (Fig. 43B), with 1µM causing approximately a 40% decrease. SN50 peptide inhibits nuclear translocation of NFκB (Abate and Schröder, 1998), and acts as a positive control to block LPS-induced nuclear translocation. SN50 dose-dependently blocked LPS-induced NFκB activity (data not shown; 20µM caused a 90% decrease), with 10µM causing approximately a 50% decrease (Fig. 43B). Further, the effect of a sub-maximal dose of SN50 (10µM) was additive when applied with 300nM nicotine. In contrast, nicotine failed to attenuate LPS-induced NFκB transactivation in wild-type SHEP1 cells (Fig.43C) lacking α4, β2 and α7 nAChRs.

Evidence suggests that LPS exposure increases PIC production in part through the NFκB pathway (Morrison and Ryan, 1987; Lazarov et al., 2000). Because nicotine blocked LPS-induced NFκB activity, we tested whether nicotinic activation of α4β2 blocks LPS-induced signaling downstream of NFκB pathway, one of which is cytokine production. Treatment of hα4β2 SHEP1 cells with 1µg/mL LPS caused an appreciable increase in both IL-1β (2-fold) and IL-6 (1.5-fold) production (Fig. 44), compared with untreated controls. By contrast, nicotine pre-treatment dose-dependently reduced LPS-induced IL-1β and IL-6 production, with 100nM nicotine causing a complete abolishment of both cytokines (Fig.44), as measured by ELISA.
Figure 43. Nicotine blocks LPS-induced NFκB activity in hα4β2.

A) Cells plated in 48-well plates were transfected with pNFκB-MetLuc2-Reporter vector overnight. Cells were then stimulated with indicated concentrations of bacterial endotoxin LPS for 4h before collecting the supernatant to measure the luciferase activity. B) Cells were pre-treated with increasing concentrations of nicotine for 2h before stimulating with 1µg/mL of LPS. A sub-maximal dose of SN50 peptide was used as a positive control. C) Wild-type SHEP1 cells were stimulated with varying concentrations of LPS for 4h with or without indicated concentrations of nicotine pre-treatment. Data represent mean ±SEM (n=3). *p<0.05, **p<0.01; ***p<0.001. RLU, relative luminescence units.
LPS-induced cytokine production involves IκBα-phosphorylation and subsequent translocation of NFκB into the nucleus (Zandi et al., 1997; Karin and Ben-Neriah, 2000). Since nicotinic activation of α4β2 receptors attenuated LPS-induced NFκB activation and thereby cytokine production, we asked whether α4β2 signaling interferes with IκBα-phosphorylation. The hα4β2 SHEP1 cells, pre-incubated with either PBS or 300nM nicotine, were stimulated with 1µg/mL LPS for 25 min at 37°C. Phospho-IκBα levels were determined by western blotting and functional ELISA. IκBα-phosphorylation was effectively attenuated by 300nM nicotine, compared with PBS treated controls (Figs.46A & 48B), indicating that nicotine opposes endotoxin inflammation by preventing IκBα-phosphorylation. Having demonstrated this, we next sought to study the signaling downstream of α4β2-receptor activation leading to inhibition of IκBα-phosphorylation.

Figure 44. Nicotine blocks LPS-induced cytokine production.

Cells plated in 6-well dishes were stimulated with 1µg/mL of LPS for 48h with or without indicated concentrations of nicotine pre-treatment. Cells were lysed and stored at -80°C until further use. Protein levels of IL-1β (A) and IL-6(B) were measured according to the manufacturer’s protocol. Data represent mean ±SEM of three independent experiments. *p<0.05, **p<0.01.

14.5. Ca2+ influx does not influence nicotine-induced cytokine suppression- The calcium permeability of α7 is comparable to that of NMDA receptors, whereas it is relatively low for α4β2 nAChRs (Dajas-Bailador and Wonnacott, 2004). To investigate the involvement of calcium, we initially tested the role of calcium using a cell permeable calcium

69
1. PBS (n=3)  
2. Methanol (n=3)  
3. 1µM A23187 (n=5)  
4. 3µM A23187 (n=5)  
5. 3µM A23187 + 3µM BAPTA (n=4)  
6. 3µM A23187 + 5µM BAPTA (n=4)  
7. 300nM Nicotine (n=6)  
8. 1µM Nicotine (n=4)  
9. 1µM Nicotine + 3µM BAPTA (n=5)  
10. 1µM Nicotine + 5µM BAPTA (n=5)

Figure 45. BAPTA pre-treatment buffers nicotine and A23187-induced increases in intracellular calcium.

Cells pre-treated with Fluo-4 AM and with or without indicated concentrations of BAPTA-AM were stimulated with A23187 or nicotine. Fluorescence was recorded at 3sec intervals for 1min using a BioTek-HT microplate reader at excitation 485±20 nm and emission 520±20 nm. 300nM and 1µM nicotine caused a significant increase, however, a 30min pre-treatment with 3µM and 5µM BAPTA blocked nicotine-induced increases in intracellular calcium. a, significantly different from controls; b, significantly different from 3µM A23187; c, significantly different from 1µM nicotine; o, indicates an outlier. Note: Box-plots were used to visualize differences among different groups. Because the data did not deviate from a normal distribution, one-way ANOVA and Tukey post-hoc tests were used to assess statistical difference (P<0.0001).
chelator BAPTA-AM on NFκB translocation and IκBα-phosphorylation. BAPTA-AM has been used extensively both in vitro and in vivo to buffer intracellular changes in calcium (Billman, 1993; Bissonnette et al., 1994). For instance, nicotine-induced increases in tyrosine hydroxylase mRNA in PC12 cells were blocked by BAPTA pre-treatment (Gueorguiev et al., 1999). We used a divalent calcium ionophore A23187 (alias calcimycin) as a positive control (Tsien et al., 1982) to verify the intracellular calcium buffering capacity of BAPTA pre-treatment at concentrations used in this study. It should be noted that the ability of A23187 to translocate calcium is several fold higher than that of nicotine and further, the calcium permeability of α4β2 is minimal (It is 4-6% compared with α7 nAChRs) (Albuquerque et al., 1997; Dajas-Bailador and Wonnacott, 2004; Wonnacott et al., 2006). Fluo-4 AM was used as the intracellular calcium indicator. Increasing concentrations of A23187 was dispensed by an automated syringe dispenser and fluorescence was monitored at 3 sec intervals for 1 min.

A23187 caused a dose-dependent increase in calcium with 3µM causing a maximum ~2 fold increase while 1µM caused a ~1.25 fold increase, compared with methanol treated controls (Fig.45). However, 30min pre-incubation with BAPTA dose-dependently buffered 3µM A23187-induced calcium flux (Fig.45), with 5µM BAPTA blocking ~50% of 3µM A23187-induced calcium influx. Further, 300nM and 1µM nicotine caused a subtle but significant increases in the fluorescent signal compared with PBS treated controls (Fig. 45), however, 3µM and 5µM BAPTA blocked this increase, suggesting that BAPTA pre-treatment effectively buffers increases in intracellular calcium due to nicotinic stimulation of α4β2 receptors.

The concentrations tested for BAPTA alone had no effect on NFκB activity in hα4β2 cells transfected with NFκB reporter vector (Fig.46A). Next, when cells were pre-treated with BAPTA-AM for 30min and further incubated with nicotine, no significant difference was observed between nicotine alone and nicotine plus BAPTA treatments (Fig.46A). Consistently, BAPTA failed to block the effects of 300nM nicotine on LPS-induced IκBα-phosphorylation (Fig.46B & C), suggesting a calcium-independent mechanism for α4β2 mediated anti-inflammatory effects.
Figure 46. α4β2-nAChR activation but not calcium flux mediates nicotine-induced anti-inflammatory effects.

A) NFκB reporter assay: Cells transfected with NFκB reporter vector were pre-treated (30 min) with 3μM and 5μM concentrations of BAPTA-AM before incubating with or without 300nM nicotine for 4h. *p<0.05.

B) Immunoblot: ha4β2 cells grown in 6-well plates were serum starved for 16h and then pre-treated (30min) with 3μM and 5μM BAPTA, and incubated with 300nM nicotine for 30min before stimulating with 1μg/mL of LPS for 25min at 37°C. Equal amounts of proteins were analyzed by SDS-PAGE, followed by electrophoretic transfer to PVDF membranes, blotting and ECL detection. The membranes were stripped and reprobed with an anti-β-actin antibody. No band was detected for control treatments.

C) phospho-IκBα ELISA: Proteins samples isolated for western blotting in panel 45B were used here. Data represent mean ±SEM (n=3).
14.6. cAMP-dependent PKA signaling- In other systems, cAMP-dependent protein kinase A (PKA) inhibits NFκB transcriptional activity (Neumann et al., 1995; Ouchi et al., 2000; Takahashi et al., 2002; Minguet et al., 2005). Although cholinergic agonists cause cAMP accumulation (Pitchford et al., 1992; Hiremagalur et al., 1993; Nuutinen et al., 2006), the involvement of cAMP signaling in nAChR-mediated anti-inflammatory effects is unknown. Our results show that nicotine and forskolin (an adenylate cyclase stimulant) cause a temporal increase in cAMP in α4β2 SHEP1 cells (Fig. 47A), with forskolin causing a maximum increase at 1h while nicotine at 4h. Therefore we initially examined the effect of forskolin and nicotine on LPS-induced NFκB translocation in presence or absence of a specific PKA peptide inhibitor (PKI 14-22 amide). Forskolin had little effect on NFκB activity and the PKA inhibitor failed to block the effects of nicotine (Fig. 47B). These results provide no evidence for the involvement of cAMP-PKA signaling in α4β2-mediated suppression of NFκB activity.

![Figure 47](image-url)

**Figure 47. cAMP-PKA pathway does not influence nicotine-induced anti-inflammation.**

A) Temporal pattern of cAMP: cells were exposed to 10µM nicotine and 10µM forskolin for indicated times, and cell lysates were used to monitor cAMP levels by ELISA.

B) NFκB reporter assay: NFκB reporter transfected cells were pre-incubated with 10µM forskolin or 300nM nicotine and/or PKA inhibitor and stimulated with 1µg/mL of LPS for 4h. a, significantly different from untreated controls; b, significantly different from LPS-alone treatment; p<0.05.
14.7. **JAK2 mediates α4β2-mediated anti-inflammation** - α7 nAChRs activate the JAK2-STAT3 pathway to attenuate endotoxin-induced inflammation (de Jonge et al., 2005; Tracey, 2007). We investigated the involvement of JAKs in α4β2 mediated anti-inflammation using a broad spectrum JAK inhibitor I and AG-490 (a specific JAK2 inhibitor) on LPS-induced NFκB translocation and an upstream IκBα-phosphorylation step. JAK inhibitor I is a very potent inhibitor of JAKs and, nanomolar concentrations of this drug robustly blocked nicotine-induced NFκB translocation in LPS stimulated cells (Fig.48a), providing a tentative evidence for a contribution of JAK in opposing LPS inflammation. Pre-incubation with 10µM AG-490 and nicotine restored NFκB translocation (Fig.48c) and IκBα-phosphorylation (Fig.48b, d) in LPS stimulated cells. Together, these results suggest that nicotine opposes LPS elicited inflammation by interfering with IκBα-phosphorylation, and JAK2 is an important intermediary in this anti-inflammatory pathway.

14.8. **NSC74859 restores LPS-induced NFκB activation** - Activated JAKs induce STATs phosphorylation, which form either homo- or hetero-dimers and translocate to the nucleus to bind promoter elements of DNA to induce gene transcription (Heinrich et al., 2003). De Jonge et al. (2005) demonstrated that nicotine dose-dependently induces STAT3 phosphorylation, and STAT3 is an essential transducer of α7-mediated anti-inflammation. We examined the possibility of STAT3 role in α4β2 SHEP1 cells by assessing the ability of a specific STAT3 inhibitor NSC74859 (Lin et al., 2009) to inhibit nicotine-induced inhibition of LPS-stimulated IκBα-phosphorylation and NFκB activity. STAT3 inhibitor dose-dependently prevented the effects of nicotine on IκBα-phosphorylation and NFκB activity, with 30µM causing a significant inhibition of both (Fig.49a & b). The STAT3 inhibitor blocked NFκB translocation, suggesting that it might be a post-transcriptional regulator of the NFκB complex, however further investigation is needed to establish definitive protein-protein interaction.
Figure 48. AG-490 blocks α4β2-mediated anti-inflammation.

a) AG-490 blocks α4β2-mediated anti-inflammation. a) NFκB reporter assay: NFκB reporter transfected cells were pre-treated (30min) with 30nM JAK I inhibitor and 300nM nicotine and stimulated with 1μg/mL of LPS for 4h. Cell supernatant was used to monitor luciferase activity. b) Immunoblot: serum starved ha4β2 SHEP1 cells were pre-treated with 300 nM nicotine and 10μM AG-490, and stimulated with 1μg/mL LPS for 25 min at 37°C. Protein samples were probed with mouse primary anti-p-IκBα or IκBα antibodies followed by a rabbit anti-HRP secondary antibody. All membranes were stripped and reprobed with a mouse anti-β-actin antibody and a rabbit anti-HRP secondary antibody. c) NFκB reporter assay: NFκB-reporter transfected cells were pre-treated with nicotine and/or varying concentrations of AG-490 and incubated with 1μg/mL of LPS for 4h. 50μL of cell supernatant was collected to assess luciferase activity. d) Phospho-IκBα ELISA: protein samples (used in figure 47b) were added to pre-coated plates and p-IκBα levels were determined according to the manufacturer’s protocol. Data represent mean ± SEM of three independent experiments. One-way ANOVA determined statistical difference among groups. a, significantly different from LPS alone; b, significantly different from LPS plus nicotine treatment (significance at 0.05 level).
Figure 49. STAT3 inhibitor blocks α4β2-mediated anti-inflammation.

a) STAT3 inhibitor blocks α4β2-mediated anti-inflammatory. a) NFκB translocation: hα4β2 cells transiently transfected with NFκB reporter vector were stimulated with LPS for 4h with or without nicotine and/or increasing concentrations of NSC74859 pre-treatment. 50µl of cell supernatant was used to measure NFκB activity (n=3).

b) phospho-IκBα ELISA: cells grown in 6-well dishes were serum starved for 16h and then pre-incubated with 300nM nicotine and indicated concentrations of NSC74859 before stimulating with 1µg/mL of LPS for 25 min at 37°C. 100µg of cell lysate was used to monitor phospho-IκBα levels by functional ELISA (n=4). Results represent mean ± SEM. a, significantly different LPS treated samples (p<0.05); b, significantly different from nicotine plus LPS treated samples (p<0.05).
15. Discussion

**α4β2-mediated neuroprotection** - Nicotine may be neuroprotective, since chronic smokers are less susceptible to neurodegenerative diseases (Cattapan-Ludewig et al., 2005; Picciotto and Zoli, 2008; Shimohama, 2009), and the incidence of smoking is very high among schizophrenics, who self-medicate to improve alertness and cognition (Cattapan-Ludewig et al., 2005; Picciotto and Zoli, 2008; Shimohama, 2009). In experimental models of MS, nicotine, administered before or simultaneous with disease induction, attenuates CNS inflammation as well as autoimmune responses (Shi et al., 2009). In experimental models of AD, nicotine administration reduces the deposition and formation of amyloid plaques (Kihara et al., 1998; Wu, 2004; Shimohama, 2009). Although substantial evidence points toward a neuroprotective evidence of nicotine (Piao et al., 2009), the underlying receptor subtypes and the molecular and cellular mechanisms are still unclear.

My findings demonstrate that concentrations as low as 100nM nicotine significantly suppress PIC production via α4β2-receptor activation. The concentration of nicotine achieved in a smoker’s brain is about 50–300 nM (Cao and Peng, 1998; Paradiso and Steinbach, 2003), which is sufficient to activate α4β2 receptors. Previous studies investigating nicotine-mediated neuroprotection suggest that chronic nicotine stimulates α4β2 receptors in the dopaminergic system and enhances neurotransmission (Penton and Lester, 2009; Xiao et al., 2009). Therefore, the putative neuroprotection seen in smokers may be due to enhanced neurotransmission and/or suppression of PICs via α4β2 nAChRs. Studies are underway to determine whether nicotine alters inflammatory cytokine expression in brains of wild-type, heterozygote and α4 knockout mice.

**α4β2-mediated anti-inflammation** -

Approximately half of the observed gene changes (19/41) in our microarray were associated with inflammatory or immune response pathways. Nicotine is anti-inflammatory, since stimulation of the vagus or nicotine administration suppresses several PICs through activation of α7 nAChRs (Tracey, 2007). Besides α7, the transcripts for other nAChR subtypes such as α3, α4, α5 and β2 have been detected in different cell types such as alveolar and peritoneal macrophages (Matsunaga et al., 2001; Dehkordi et al., 2008; Gu et al., 2008; Osborne-Hereford et al., 2008; van der Zanden et al., 2009). Thus, an important question is whether α4β2 nAChRs, the major subtype for high affinity binding in the CNS, can also mediate anti-inflammatory effects of nicotine. Matsunaga et al. (2004) and Van der Zanden et al. (2009) suggest that nicotine down-regulates production of PICs in macrophages expressing α4/β2. We
found that nicotine suppresses PICs IL-1β and IL-6 in hα4β2 SHEP1 cells in a time- and concentration-dependent manner. Further, selective antagonists dHβE and Mec reversed nicotine-induced suppression of PICs. A similar set of experiments in wild-type SHEP1 cells did not detect similar changes, indicating that α4β2 nAChRs are required for this effect. Nicotine signaling through α7 receptors regulates tumor necrosis factor alpha and NFκB at a post-transcriptional level (de Jonge et al., 2005). Nicotine did not alter expression of either gene measured by qPCR in our system (Table 1), but nicotine-mediated suppression of PICs through α4β2 receptors modulates NFκB activation. Moreover, α4β2 antagonists reversed nicotine-induced suppression of PICs and NFκB transactivation, suggesting that receptor activation and signaling is required to inhibit activation of the NFκB pathway and cytokine production.

The anti-inflammatory effects of α7 are mediated via inhibition of NFκB (Wang et al., 2004) and/or activation of JAK2-STAT3 signaling (de Jonge and Ulloa, 2007), and these responses can be blocked by selective α7 antagonists (Borovikova et al., 2000; de Jonge et al., 2005). We examined whether JAK2-STAT3 signaling was involved in suppression of LPS-induced inflammatory responses such as PIC production and NFκB activity.

**Resemblance to anti-inflammatory IL-10 receptor signaling.**

IL-6 receptor (IL-6R) stimulation leads to tyrosine phosphorylation of its receptors and activation of the cytoplasmic transcription factor STAT3 (Akira et al., 1994; Taga and Kishimoto, 1997). Phosphorylated STAT3 forms dimers and translocates to the nucleus and induces SOCS3 expression (Schindler et al., 2007). Increased SOCS3 functions to attenuate PIC (IL-1β and IL-6) production. Moreover, SOCS3 is a negative regulator of IL-6R-STAT3 signaling (Alexander and Hilton, 2004).

Conversely, although IL-10R activation of JAK2-STAT3 is critical for the anti-inflammatory actions of IL-10 (Asadullah et al., 2003), IL-10R signaling inhibits LPS- and TNFα -induced PIC production by preventing IκBα phosphorylation, NFκB activation and NFκB DNA-binding activity (Wang et al., 1995; Schottelius et al., 1999), but not through increased SOCS3 expression (Kubo et al., 2003; Lang et al., 2003). This suggests that IL-10 suppresses PIC production through a non-transcriptional mechanism. Similar to IL-10R signaling, we found that α4β2 activation suppresses LPS-induced effects by preventing IκBα phosphorylation and NFκB translocation. De Jonge et al. (2005) showed that siRNA-mediated depletion of SOCS3 expression does not affect cholinergic anti-inflammatory responses in peritoneal macrophages. Likewise, Wang et al. (2004) suggest that nicotine inhibits TNFα-induced HMGB1 release in human macrophages by inhibiting the NFκB pathway, without altering the message or protein levels of HMGB1.
Together these findings suggest that the nAChR-mediated anti-inflammatory pathway resembles IL-10 receptor signaling. Although Wang et al. (2004) and De Jonge et al. (2005) implicated a non-transcriptional mechanism for the cholinergic anti-inflammatory pathway, the underlying mechanism is unknown. Our results show that a highly specific STAT3 inhibitor blocks IκBα-phosphorylation and thereby inhibiting NFκB translocation and PIC production. This is the first indication that STAT3 inhibits NFκB activity through a non-transcriptional mechanism upon α4β2 activation, and strongly supports the previous hypotheses that cholinergic stimulation prevents NFκB activity (Wang et al., 2003; Ulloa, 2005) and mimics IL-10 receptor signaling (De Jonge et al., 2005). Further investigation will need to establish the interaction between STAT3 and NFκB.

Evidence for calcium and cAMP-PKA independent anti-inflammatory signaling-

Nicotinic acetylcholine receptor-mediated calcium flux induces several physiological changes by activating diverse signaling pathways such as cAMP-PKA, MAPK and PI3K (Berg and Conroy, 2002; Hu et al., 2002; Brunzell et al., 2003; Dajas-Bailador and Wonnacott, 2004). By activating these pathways, nicotine regulates the expression of genes including cAMP response element binding protein, tyrosine hydroxylase and Bcl2 (Ishiguro et al., 1997; Hu et al., 2002; Shimohama, 2009). We investigated the involvement of calcium and found that nicotine-induced increases in calcium flux through α4β2 receptors had no effect on α4β2-mediated NFκB activity. This result is in agreement with a previous finding that human leukocytes expressing nAChRs fail to elicit detectable currents in response to cholinergic agonists nicotine and acetylcholine, although both agonists induce anti-inflammatory actions, suggesting a plausible role for calcium-independent signaling (Villiger et al., 2002). It is possible that an agonist-induced conformational change in α4β2 nAChR could promote nicotine-induced immunosuppressive and anti-inflammatory effects. Conversely, there could be yet unidentified nAChR-scaffolding or anchoring proteins that facilitate the signal transduction.

Previous studies in different culture systems showed that agents that elevate intracellular cAMP inhibit IκBα-phosphorylation and subsequent NFκB activation (Takahashi et al., 2002; Sands et al., 2004; Minguet et al., 2005). Adiponectin-induced accumulation of cAMP in human aortic endothelial cells prevents TNFα-induced IκBα-phosphorylation and NFκB activation (Ouchi et al., 2000). Further, this effect is blocked by both adenylate cyclase inhibitors as well as PKA inhibitors, implicating a convergence of cAMP-PKA and NFκB signaling pathways. In
contrast, we found that although forskolin and nicotine caused cAMP accumulation, forskolin failed to restrict LPS-induced NFκB activation, and further a highly specific PKA inhibitor failed to block the actions of nicotine in α4β2 cells. These data demonstrate no evidence for the role of cAMP-PKA dependent pathway in α4β2-mediated suppression of NFκB activation in our system. Further studies need to examine why cAMP-PKA anti-inflammatory signaling is restricted to certain receptor types, regardless of considerable cAMP accumulation.

A ‘functional desensitized-receptor signaling’ hypothesis-

Nicotinic acetylcholine receptors are ligand-gated ion channels in the Cys-loop superfamily (Claudio et al., 1987; Lindstrom et al., 1990). Five homo or hetero subunits assemble to form a functional receptor. Each subunit has four transmembrane domains. The ligand-binding domain is located in the extracellular region of the receptor and the ion channel is in the transmembrane domain (see Fig.50A). The receptor could exist in different states:

1. Open- agonist bound and conducting state
2. Desensitized- agonist bound and non-conducting (high-affinity state)
3. Closed- absence of agonist

The C-loop denotes the configuration of the channel (Fig.50B). Agonist binding shifts the configuration of C-loop, β1- β2 linker and β8- β9 linker in a clock-wise direction, possibly due to agonist-induced receptor activation. The C-loop in a ‘closed’ configuration reflects both the ‘open’ and ‘desensitized’ states of the receptor. However, the C-loop ‘open’ configuration indicates the ‘closed’ (resting) state of the receptor channel (Gay and Yakel, 2007).

Continuous exposure to low nicotine concentrations (nM concentrations used in this study), can induce desensitization without receptor activation, a process referred to as ‘high-affinity desensitization’(Giniatullin et al., 2005). Although the mechanism of desensitization is unclear, research suggests that α4/β2 genes encoding mutations in M2-transmembrane alter receptor desensitization and channel function (Bertrand et al., 2002). Chronic smokers experience sustained exposure to low concentrations of nicotine (~100nM) and it is possible that the desensitized (non-conducting) receptors rather than activated (conducting) receptors confer neuroprotection. We propose a ‘functional desensitized signaling’ hypothesis because blockade of nicotine-induced calcium influx by BAPTA did not alter the anti-inflammatory actions of nicotine. It is possible that JAK2-STAT3 signaling might be associated with the desensitized or non-conducting state of α4β2 receptor. The C-loop is in a closed configuration in the desensitized state
of the receptor, and a slight rotation of C-loop, due to agonist binding, could induce conformational changes in intracellular loops 1 and 2.

Figure 50. Ribbon representation of two Torpedo nicotinic acetylcholine receptor subunits -

A) Topology of two subunits- The ligand-binding domain is on the extracellular side. The transmembrane domain has the aromatic pocket that forms a binding site for agonist (Xiu et al., 2009). α-helices are indicated in red and β-strands in blue.

B) The ligand-binding domain is comprised of a transmitter binding site and a transition zone. The aromatic binding pocket is located in the transmitter binding site and the C-loop acts as a cap. Note: C-loop configuration reflects the open/desensitized/closed states of the receptor. The transition zone is comprised of the Cys-loop, β8-β9, β1-β2, β10-M1 and M2-M3 linkers.

C) Agonist binding causes a rotation at the transmitter site that passes through to the Cys-loop, β1-β2 and β8-β9 loops. Unwin’s model (2003) of gating suggests that the C-loop and β1-β2 loop rotate in a clock-wise direction around an axis. C-loop, Cys-loop, β1-β2 loop and β8-β9 loop are indicated by a red dotted circle. Modified from (Gay and Yakel, 2007).
Possible cytoplasmic domains involved in JAK2 and STAT3 activation- Our results demonstrate that highly specific JAK2 and STAT3 inhibitors completely antagonize the anti-inflammatory effects of nicotine by preventing IkBα phosphorylation and NFκB activation. This is an early indication that JAK2-STAT3 might be involved in α4β2-mediated anti-inflammatory effects. Further, an important finding of this study is that STAT3 retains NFκB in the cytoplasm and prevents its activation. Yu et al. and others have shown that the DNA-binding domain of STAT3 physically interacts with p65 subunit of NFκB and prevents its translocation (Yu et al., 2002; Yu and Kone, 2004; Hoentjen et al., 2005). We speculate that similar mechanisms might attribute to the anti-inflammatory effects of α4β2 activation. However, further studies will require elucidating specific regulation of NFκB complex by activated STAT3 protein. Further, it is possible that since both α7 and α4β2 attenuate endotoxin-induced inflammation through JAK2-STAT3 signaling, there might be nAChR-specific anchoring or scaffolding proteins that are sensitive to conformational changes in the receptor and serve to transduce signals to JAK2 activation.

Using western blotting and immunoprecipitation studies, De Jonge et al. (2005) demonstrated that JAK2 interacts with α7 receptors in peritoneal macrophages, which is required for STAT3 activation. However, domains of α7 interacting with JAK2 are unknown. Our findings suggest a calcium-independent signal transduction leading to speculation about conformational changes in the receptor to cause JAK2 activation. This raises several important questions: 1) How is the signal transduced from the receptor to JAK2? 2) Does a conformational change in the intracellular loop lead to JAK2 activation? 3) Does the α4 subunit or the β2 subunit or both mediate JAK2 activation? 4) Does the open or the desensitized state of the receptor favor JAK2 binding? 5) Is the immune modulatory activity restricted to α4β2 and α7 or does it apply to other ligand-gated ion channel receptors?

Although both α7 and α4β2 seem to employ JAK2-STAT3 signaling, surprisingly, there is little homology in the central portion of the major M3-M4 loop (Fig.51). Homology is restricted to the first intracellular loop M1-M2 (ICL 1) and the beginning (ICL 2A) and the end of M3-M4 loop (ICL 2B). Previous studies suggest that while STAT3 phosphorylation is dependent on a single tyrosine residue of the receptor, there is no consensus binding motif for JAK2 activation (Behrmann et al., 1997; Ahr et al., 2005).
Figure 51. Schematic representation of nAChR subunit and putative sites for JAK2 binding and STAT3 activation.

The nAChR subunit is a four transmembrane structure with both N- and C-terminals facing the extracellular side. The blue loops represent areas of conserved residues between various ligand-gated ion channel (LGIC) receptors. The intracellular loop 1 of various LGIC receptors has a highly conserved proline (arrow), glycine (arrow head) and glutamic acid (arrow head), which might be involved in JAK2 binding. On the other hand, the ICL-2A, rich in prolines and other non-polar amino acids, could also serve as a JAK2 binding site.

The ICL-2B of α4 has two YXX motifs: YIA and YVA, whereas α7 has the YIA and β2 has YVA motif. The YXX (X= non-polar residues; L/I/V) serves as a docking site for STAT3 in various cytokine and chemokine receptors. The YIA of α7 and YVA of α4/β2 could be involved in STAT3 docking and phosphorylation.

Abbreviations: ICL, intracellular loop; P, proline; G, glycine; E, glutamic acid; K, lysine; H, histidine; T, threonine; W, tryptophan; L, leucine; V, valine; I, isoleucine; Y, tyrosine; A, alanine; D, aspartic acid; R, arginine; N, asparagine; C, cysteine; Q, glutamine; M, methionine; F, phenylalanine; S, serine; Underlined amino acids are non-polar. Note: Sequences for α3, β4, 5HT3A and 5HT3B are included to compare α4/β2 and α7 with other nAChR subunits and cationic LGIC receptors. Nicotinic receptor subunit image is modified from (Karlin, 2002).
An intracellular SHSK motif located on the intracellular loop 3 of CXCR4 (a G-protein coupled receptor) activates JAK2 (Fig.38), whereas a highly conserved glycine, box1 and box2 of the erythropoietin receptor (a cytokine receptor) are essential for JAK2 biological activity (Fig.37). Future investigation will need to examine which intracellular binding domains of α4β2 and α7 nAChRs are involved in JAK2 activation.

In contrast, the motif YXX (X= non polar residues; L/I/V) of various cytokine and chemokine receptors is hypothesized to act as a binding site for STAT proteins (Songyang et al., 1993; Gauen et al., 1994; Ahr et al., 2005). For example, this motif is conserved among the GPCRs (eg.CXCR4 and CCR2b) (Rodriguez-Frade et al., 1999). Recently, Charpantier et al. (2005) examined the role of tyrosine phosphorylation (Tyr-386 and Tyr-442) of α7 receptors in SH-SY5Y cells and report that replacement of Tyr-386 and Tyr-442 by a non-polar Alanine makes α7 receptors more active than wild-type receptors (Charpantier et al., 2005). Our sequence homology analysis indicates that α7 and α4 have two and three conserved tyrosine residues, respectively, in the intracellular loop 2B (ICL-2B), whereas β2 has a single conserved tyrosine residue in ICL-2B (Fig. 51). Further, NetPhos 2.0 [a computer-based algorithm to predict serine, threonine and tyrosine phosphorylation sites in independent sequences (Blom et al., 1999)] program predicted Tyr-442 of human α7 (0.964*), and Tyr-462 of human α4 (0.970*) and β2 (0.970*) subunits as high potential sites for tyrosine phosphorylation (Fig.52). Based on these data, we propose that the YIA motif of α7 and the YVA motif of α4 and β2 (Fig.51) might be involved in nAChR-mediated STAT3 phosphorylation.
Comparison of possible tyrosine phosphorylation sites in the intracellular loop 2B: α4/β2 versus α7

Figure 52. NetPhos 2.0 predicted sites for serine, threonine and tyrosine phosphorylation in the ICL 2B (437-469) of α7, α4 and β2 nAChR subunits:

A) The α7 intracellular loop 2 has two conserved tyrosine residues: Tyr-386 and Tyr-442. The y-axis is representative of phosphorylation potential (score ranges from 0.0 to 1.0). 0.5 is the threshold for phosphorylation potential and higher the score higher is the possibility of phosphorylation. A score below 0.5 indicates that the possibility of phosphorylation of that residue is very low. In this case, a score of 0.964 for Tyr-442 indicates a higher probability of phosphorylation (Charpantier et al., 2005).

B) The intracellular loop 2 of α4 has three conserved tyrosine residues (only two residues between 437 and 469). Tyr-462 has a greater possibility, while the score for other two residues was around 0.25, implicating a very low possibility.

C) The intracellular loop 2 of β2 has a single tyrosine residue at position 462, which has a very high
In summary, α4β2-mediated anti-inflammatory signaling is an important discovery of this study and advances our understanding of how chronic smoking could confer neuroprotection against neurological disorders. The elucidation of JAK2-STAT3 and NFκB signaling pathways aids in developing new therapeutic targets against neurodegenerative diseases such as AD and PD.

The α4β2-nAChRs mediate previously unknown anti-inflammatory effects via JAK2-STAT3 signaling, which prevents IκBα phosphorylation, NFκB translocation and PICs IL-1β and IL-6 production. Evidence points toward a non-calcium and cAMP-PKA independent pathway. The α4β2-agonists dHβE and Mec block nicotine-induced decreases in NFκB translocation and cytokine production. The α4β2-mediated suppression of pro-inflammatory cytokines may explain the neuroprotective effects of nicotine observed in smokers. Further investigation will need to examine how activated STAT3 inhibits NFκB transactivation and more importantly, how the signal is transduced from α4β2 to JAK2. (Modified from SABiosciences pathway, SABiosciences Corporation).
16. Future Directions

Here we provide an early evidence for the involvement of α4β2-mediated suppression of inflammation against the endotoxin LPS exposure. We provide evidence against the involvement of calcium and cAMP-dependent PKA pathway, and show evidence to support the convergence of JAK2-STAT3 and NFκB pathways in α4β2-mediated immunosuppressive and anti-inflammatory effects. Microglia are the first line of defense in the CNS. An important question would be to test whether microglia and other immune cells of the CNS express functional α4β2 nAChRs. Further, to address the involvement of similar signaling cascades, specific JAK2 and STAT3 inhibitors could be used to assess the effects of nicotine and other cholinergic agonists on LPS-induced endotoxemia, by monitoring the NFκB activity.

Most important, is another question: whether α4β2 receptors recruit JAK2 to initiate STAT3 phosphorylation and thereby prevent NFκB translocation. Our findings support the involvement of JAKs, especially JAK2, because two different pharmacological agents, a promiscuous JAK inhibitor I and a specific JAK2 inhibitor AG-490, prevented the anti-inflammatory effects of nicotine. Future studies should address whether activation of α4β2 nAChRs recruits cytoplasmic JAK2. Immunoprecipitation assays can be used to test the possible association between α4β2 receptors and JAKs. Further, mutational analyses can be performed in different motifs of intracellular loops of α4 and β2 subunit to investigate which subunits and domains contribute to JAK2 activation and STAT3 phosphorylation. NFκB translocation and IκBα phosphorylation can be determined after transfecting cells with mutated α4 or β2 subunits.

Although a highly specific STAT3 inhibitor was used to demonstrate that JAK2-STAT3 prevents IκBα - phosphorylation and subsequent NFκB activation, the underlying interaction between STAT3 and NFκB complex needs further investigation. To address this, the following experiments need be performed: SH2 domain of STAT3 is implicated to interact with p65 subunit of NFκB (Yu et al., 2002; Yu and Kone, 2004; Hoentjen et al., 2005). Constructs expressing a deleted or mutated SH2 domain can aid in addressing whether protein-protein interactions are involved between STAT3 and NFκB, and if so, which domains are imperative for this interaction. Alternatively, similar studies could be performed utilizing mutated NFκB constructs. Together, these results will shed light on the key link between two divergent pathways: JAK2-STAT3 and NFκB.
Identification of α4/β2 cytoplasmic domains required for JAK2 binding-

Previous literature does not identify a consensus binding site for JAK2 across multiple receptor families. However, genetic and biochemical studies of cytokine receptors supports the role for juxtamembrane [consisting of conserved non-polar amino acids leucine (Leu), isoleucine (Ile) and tryptophan (Trp)], box 1 [conserved proline (Pro) and glycine (Gly)] and box 2 [conserved Leu, valine (Val), glutamic acid (Glu) and serine (Ser)]. Sequence homology of α4/β2 (Fig.51) indicates that the ICL-2 of α4 and β2 is rich in Pro and Gly amino acids, and also has a high degree of conserved non-polar amino acids including Trp, Leu and methionine (Met). Single-point mutations or a series of replacements of non-polar amino acids in α4/β2 can address whether these conserved residues bind and/or activate JAK2.

Mutant α4/β2 constructs can be transfected transiently into cell lines such as wild-type SHEP1 or HEK293 and the biological activity of JAK2 can be assessed by determining the ability of nicotine to induce NFκB translocation or IκBα phosphorylation. Green fluorescent protein (GFP) can be used as a positive control to estimate the transfection efficiency. [H]-cytisine binding assays need to be performed to examine whether the mutant constructs assemble properly to form functional receptors. Alternatively, α4β2-mediated suppression of NFκB translocation can be used as a functional assay to examine whether mutant constructs assemble into functional receptors.

In addition, molecular modeling, bioinformatics studies and protein structural analysis are required to understand the topology of intracellular loops 1 and 2. It should be noted that α4β2 nAChR is a pentamer and there might be a possibility of tyrosine trans-phosphorylation. For example, if the ICL-1 is involved in JAK2 binding/activation, and because the putative tyrosine (Tyr) phosphorylation site required for STAT3 docking is in the ICL-2B, the topological positioning of ICL-1 and ICL-2B might not facilitate Tyr-phosphorylation by JAK2. Conversely, since α4β2 is a pentamer, JAK2 bound to ICL-1 of one receptor subunit might trans-phosphorylate Tyr in ICL-2B of an adjacent receptor subunit (see Fig.53). Molecular modeling will aid in addressing some of these questions.
Identification of STAT3 docking site on α4β2 receptors-

Research suggests that STAT3 activation is dependent on a single tyrosine. The motif YXX (X=non-polar amino acids) serves as a STAT3 binding site for various cytokine and chemokine receptors (Ahr et al., 2005; Pelletier et al., 2006). NetPhos 2.0 predictions and sequence homology of nAChR subunits implicate that Tyr-462 (see Fig. 52) followed by three non-polar amino acids (Met, Val, Ile) in the ICL-2B of α4 and β2 could serve as a docking site for STAT3. It should be relatively easier to identify STAT3 binding sites compared with that of JAK2 because the ICL-2 of α4 has three conserved Tyr residues whereas β2 has only one (Tyr-462) (Fig.51). Single-point mutation of Tyr-462 will address whether YVA motif of α4/β2 serves as a binding site for STAT3. Mutated α4/β2 constructs can be transfected into cell lines and the biological activity of STAT3 can be assessed by determining the ability of nicotine to inhibit NFκB activity.

Figure 53. Does the orientation or topology of the cytoplasmic domains favor trans-phosphorylation?

α4β2 nAChR is a pentamer and the ICL-1 has a putative JAK2 binding/activation site, and the putative tyrosine (Tyr) phosphorylation site required for STAT3 docking is located in the ICL-2B, which leads to speculations about trans-phosphorylation rather than auto-phosphorylation.

Note: The image shows two nAChR subunits. Modified from (Karlin, 2002).
Do PICs contribute to nicotine-induced upregulation or does upregulation lead to suppression of PICs?

The sequence of events from the nicotine time-course experiment (Microarray analysis) suggests that an 8h nicotine exposure suppresses PIC production while increasing surface α4β2 receptor expression. Subsequent studies, using western blotting and ELISA, indicate that nicotine-induced immune suppressive (NFκB activity- 4h exposure) and anti-inflammatory (IkBα phosphorylation- 30min pre-treatment before stimulation with LPS for 25min) effects occur much earlier than observed increases in α4β2 surface receptors, suggesting that α4β2 upregulation could be outcome of immune suppression (IL-1β and IL-6) (Fig.54). This finding raises several questions about the physiological regulation of immune responses by cholinergic activity and vice versa. Activation of nAChRs by cholinergic agonists attenuates PIC production (Tracey, 2007), however, an important question is whether PICs govern the expression of nAChRs for maintenance of physiological homeostasis. Gahring et al. (2005) tested the influence of PICs IL-1β and TNFα on association of α4, β2 and β4 subunits in HEK293 cells and report that PIC-exposure alters the assembly of nAChRs. Together, these studies imply a negative association between induction of ER chaperones and PIC production. However, it is unlikely that immune suppression could trigger α4β2 upregulation because antagonists such as Mec and dHβE elicit increases in α4β2 receptor expression, nonetheless, do not affect immune responses observed when nicotine is administered. Further investigation is needed to examine the influence of immune suppression on α4β2 upregulation.

![Diagram of immune suppression and nAChR assembly](image)

Figure 54. Does immune suppression trigger α4β2 upregulation?
Our findings point to a negative correlation between $\alpha_4\beta_2$ receptor upregulation (also an increase in ER chaperone expression) and PIC downregulation, which could account for the following two puzzles:

1. Loss of $\alpha_4\beta_2$ nAChRs observed in patients with neurological disorders, manifested by excess inflammation (Perry et al., 2001; Lee et al., 2002; Bourin et al., 2003; Martin-Ruiz et al., 2004; Pimlott et al., 2004; Ripoll et al., 2004). In this case, PICs might be altering the assembly of nAChR subunits.

2. While positron emission tomography (PET) and post-mortem studies of a chronic smoker’s brain reveals an appreciable increase in high-affinity binding $\alpha_4\beta_2$ sites for nicotine (Wullner et al., 2008), consistent studies reveal lower inflammatory cytokines (IL-2 and IL-6) in chronic smokers compared with their non-smoking counterparts (Zhang et al., 2008).

**Conclusion**

Microarray analysis showed that chronic nicotine subtly alters gene expression of cytokines, chemokines, transcription factors, possible ER chaperones and cytosolic proteins in h$\alpha_4\beta_2$ SHEP1 cells. Induction of ER chaperones and cytosolic proteins after initial increases in $[^3H]$cytisine binding suggests a plausible negative feedback on $\alpha_4\beta_2$ upregulation triggered by nicotine, although further investigation is required to elucidate distinct functions of individual genes contributing to $\alpha_4\beta_2$ receptor expression. By use of correlation analysis and RNA interference we provide early evidence that ER proteins, such as CRELD2, negatively regulate $\alpha_4\beta_2$ surface receptor expression. Another important finding of this study is that nicotinic activation of $\alpha_4\beta_2$ receptors mediates immune suppressive and anti-inflammatory effects, which may explain the neuroprotective effects observed in chronic smokers against neurological disorders, such as AD and PD.
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