ABSTRACT

NAJLA TASLIM. ROLE OF NICOTINIC ACETYLCHOLINE RECEPTOR SUBTYPES $\alpha_4\beta_2$ AND α_7 IN NICOTINE-ETHANOL INTERACTION AND CROSS-TOLERANCE: FUNCTIONAL CORRELATION WITH CEREBELLAR NITRIC OXIDE. (Under the Direction of Dr. M.S. Dar) Department of Pharmacology and Toxicology, June 2010

The prevalent co-abuse of alcohol and tobacco products results in higher morbidity and mortality rates than those caused by either drug alone, thus posing a serious health problem. Understanding the interactive relationship between nicotine and ethanol might lead to effective strategies for the treatment of co-addiction. This dissertation project was designed to investigate the role of two nicotinic-acetylcholine-receptor (nAChR) subtypes (i.e. $\alpha_4\beta_2$ and α_7) on nicotine's attenuation of ethanol ataxia and its functional correlation with alterations in cerebellar nitric oxide concentration. Expression levels of cerebellar $\alpha_4\beta_2$ and α_7 subtypes were also determined immunohistochemically.

Ethanol (2g/kg;i.p.)-induced ataxia was assessed by Rotorod in stereotaxically cannulated CD-1 male mice following the acute or repeated intracerebellar (ICB) microinfusions of $\alpha_4\beta_2$- and α_7-selective agonists, RJR-2403 and PNU-282987. Acute RJR-2403 (31, 62, 125ng), and PNU-282987 (25ng, 250ng, 2.5µg), dose-dependently reduced ethanol ataxia. Pretreatment with potent $\alpha_4\beta_2$ [Dihydro-β-erythroidine (DHβE)] and α_7 [Methyllycaconitine (MLA)]-subtype–selective antagonists prevented RJR-2403 and PNU-282987's attenuation of ethanol ataxia, respectively. Both antagonists also offset nicotine's reduction of ethanol ataxia, confirming the contribution of $\alpha_4\beta_2$ and α_7 subtype in ethanol ataxia. There was no tonic role of either subtype in ethanol ataxia. Additionally, ICB $\alpha_4\beta_2$ and α_7 subtype antisense treatment data correspond with agonist-induced behavioral responses. Animals repeatedly microinfused with RJR- 2403 or PNU-282987 became tolerant to ethanol ataxia. The observed cross-tolerance was faster in onset and longer in duration with PNU-282987 than RJR-2403. Pretreatment with DHβE and MLA, prevented the development of cross-tolerance.

The cerebellar nitrite+nitrate (NO_x) levels were significantly enhanced and reduced following acute/repeated RJR- 2403/PNU-282987 microinfusion and acute ethanol

injection, respectively. Pretreatment with RJR- 2403 or PNU-282987 followed by ethanol prevented the ethanol-induced decrease in NO_x concentration in both acute and repeated treatment paradigms, thus correlating the decrease in NO_x concentration with ataxia and elevation with attenuation of ataxia. Both $\alpha_4\beta_2$ and α_7 subtypes exhibited high immunoreactivity in Purkinje, however, expression in molecular and granular cell layers was sparse. Overall, the results of the project support the role of $\alpha_4\beta_2$ and α_7 subtypes in the functional interaction between nicotine and acute ethanol ataxia, with NO-cGMP signaling as a participating factor.

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A Dissertation Presented to

The Faculty of the Department of Pharmacology and Toxicology

East Carolina University-Brody School of Medicine

In partial Fulfillment

Of the Requirements for the Degree

Doctor of Philosphy in Pharmacology and Toxicology

By

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June 2010

ROLE OF NICOTINIC ACETYLCHOLINE RECEPTOR Subtypes $\alpha_4\beta_2$ AND α_7 IN NICOTINE-ETHANOL INTERACTION AND CROSS-TOLERANCE: FUNCTIONAL CORRELATION WITH CEREBELLAR NITRIC OXIDE

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ACKNOWLEDGEMENTS

First of all, I am immensely thankful to my mentor, Dr. M. Saeed Dar, for his continual guidance, kind support and attention. His invaluable directions guided me throughout my experiments as well as manunscript and thesis- writing processes. Without his persistance and backing, I would not be able to publish my first manuscript within the very first year of my lab work.

Secondly, Dr Ken Soderstrom deserves most credit for providing me with his feedback, stimulating ideas and resolving scientific challenges that I encountered in my work. He also generously extended all laboratory resources and technical support to me for my project. My utmost gratitude goes to Dr David Taylor (Chair of Pharmacology & Toxicology), Dr Rukiyah Van Dross and Dr Richard Ray for their suggestions and comments and for meticulously editing my thesis for improvement. I specially appreciate Dr Taylor, Dr James Gibson and Dr McMillen for their upbeat, supportive and motivational attitude towards graduate students that serves to promote productivity and creativity.

Dr Chun-xiao Li, Weixie and Hoda Khafaga have been invaluable in providing technical training and assistance, any time I needed them. I am also thankful to Dr Richard Franklin (Department of Microbiology and Immunology) for his assistance and generosity in allowing me to use Fluorometer for nitric oxide assay and Mr. Alan Branigam for helping in generating the computer illustration for this project.

I tremendously owe my friends and colleagues Brandy Riffle and Hercules Maguma for their relentless and indescribable support, love and sincerity that led to excellent camaraderie among us. They along with, Dr. Pat Dunn (Greenville Mayor), Mr.

and Mrs. Gene McCreary, Dr. Ryan, Mrs. Dar and Dr. Ahmed Salahuddin created a family-like atmosphere for me here in the United States. Without them, I couldn't image, how would I have coped with the entirely new culture, system and the environment. I have never seen such a selfless, benevolent, utterly dedicated and spiritually upright person as Dr Dunn in my entire life. May our LORD bless her and the others.

I also express my genuine gratitude to the Department of Pharmacology, and Graduate School, particularly, Dr George Kasperek and Katrina Searcey, for their commendable efforts in providing me with all the resources to accomplish my goals here at the Brody School of Medicine.

Finally, I am undeniably, most grateful and obliged to my LORD, ALLAH subhano watalah for his boundless mercy, impeccable guidance and illuminating my life with unwavering spiritual strength that carried me through all the challenges in my life.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

aCSF	Artificial cerebrospinal fluid
AMPA	(RS)-α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance;
AP	Anterioposterior
AUC	Area under the curve;
DAN	2,3-diaminonaphthalene;
DCN	Deep cerebellar nuclei
DHβE hydrobromide	Dihydro-β-erythroidine hydrobromide
DMSO	Dimethyl sulfoxide;
DV	Dorsoventral
G-6-P	Glucose 6-phosphate;
G-6-PDH	Gglucose-6-phosphate dehydrogenase;;
I.C.V.	Intracerebroventricular
ICB	Intracerebellar
iNOS	Inducible nitric oxide synthase
MK-801	(5R,10S)-(-)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate
ML	Mediolateral
MLA	Methyllycaconitine
nAChRs	Nicotinic acetylcholine receptors

NBQX	2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide
NMDA	N-Methyl-D-aspartate
NO	Nitric oxide
NO_x	total sum of nitrite + nitrate
ODQ	1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one
PNU-282987	N-(3R)-1-Azabicyclo[2-2-2]oct-3-yl-4-chlorobenzamide
RJR-2403	(E)-N-Methyl-4-(3-pyridinyl)-3-buten-1-amine
S.C.	subcutaneous
SMT	(S)-Methylisothiourea sulfate

CHAPTER ONE: INTRODUCTION

General Introduction and Purpose of the Project

Nicotine and ethanol are the most widely consumed psychoactive drugs worldwide. Epidemiological studies reported that 80-90% of alcoholics regularly smoke tobacco (Pomerleau et al., 1997; Romberger and Grant, 2004). Similarly, the consumption of alcohol is 10 times more prevalent in tobacco smokers than nonsmokers (DiFranza and Guerrera, 1990). This strongly suggests that those who abuse these drugs may be more vulnerable to ethanol and nicotine addiction than non-abusers. It is also evident that a strong functional relationship may exist between the two drugs. The societal cost of the compulsive co-abuse of these drugs is in billions of dollars to treat smoking related morbidities as well as ethanol associated illnesses. The grave consequences resulting from the co-addiction of nicotine and ethanol necessitates an understanding of the underlying mechanisms in the functional interaction to enable possible development of effective therapeutic intervention. Because epidemiological data provided strong circumstantial evidence for a functional interaction between these drugs, we thought it would be interesting to investigate motor behavioral consequences of co-administration of nicotine and ethanol in a male CD-1 mouse model. Within the central nervous system, functional interactions between nicotine and ethanol have been well documented (Hays et al., 1999; Anthony et al. 2000; Meyerhoff et al., 2006). However, the precise pharmacological mechanism(s) of interaction between nicotine and ethanol are not well understood and appear complex. Previously, we have demonstrated that direct microinfusion of intracerebellar (ICB) nicotine markedly antagonizes ethanol-induced motor

impairment dose-dependently in a glutamate-nitric oxide-cGMP sensitive manner. Interestingly, repeated ICB microinfusion of nicotine completely abolished acute ethanol ataxia indicating development of cross-tolerance between the two psychoactive drugs (Al-Rejaie and Dar, 2006b, c). This dissertation research, therefore, represented a logical extension of our previous work and was aimed to determine the role of specific nicotinic nAChRs subtypes i.e. $\alpha_4\beta_2$ and α_7 subtypes in the functional interaction between acute/chronic nicotine and alcohol-indcued ataxia as the test response and possible participation of cerebellar NO signaling.

The cerebellum was selected for studying the nicotine and ethanol interaction because it is the key motor brain area for coordinating motor activity and an important site for the action of nicotine to attenuate ethanol-indcued ataxia (Al-Rejaie and Dar, 2006 a,b). A number of nAChR subtypes such as $\alpha_3\beta_2$, $\alpha_4\beta_2$, $\alpha_3\beta_4$, $\alpha_4\beta_4$, α_6, α_5 and most notably $\alpha_4\beta_2$ and α_7 have been detected in the cerebellum in humans and rodents (Graham et al., 2002; Turner and Kellar, 2005). Review of the current literature favors a predominant role of $\alpha_4\beta_2$ and α_7 subtypes in mediating several behavioral actions of nicotine (Damaj et al., 1995a; Picciotto et al., 1998; Chan et al., 2007) and ethanol (Le et al., 2000; Bowers et al., 2005; Steensland et al., 2007). Therefore, this constituted the primary reason for the hypothesis that nAChR $\alpha_4\beta_2$ and α_7 subtypes are involved in the behavioral interaction between acute nicotine and ethanol, the development of cross-tolerance between chronic nicotine and acute ethanol ataxia and their overall functional correlation with the cerebellar nitric oxide (NO) concentration**.** Support for this hypothesis was provided when direct ICB microinfusion of acute nicotine or agonist of nAChR $\alpha_4\beta_2$- and α_7-selective subtypes markedly and dose-dependently attenuated ethanol-induced motor impairment. The

attenuation of ethanol ataxia by ICB nicotine, the $\alpha_4\beta_2$-selective agonist, RJR-2403, and the α_7-selective agonist, PNU-282987, was virtually abolished following ICB pretreatment with DHβE, an $\alpha_4\beta_2$-selective antagonist, and methyllycaconitine, an α_7-selective antagonist, respectively. This observation confirmed the participation of the nAChR subtypes, $\alpha_4\beta_2$ and α_7, in the functional interaction between acute nicotine and ethanol ataxia. Intracerebellar treatment with antisense oligonucleotides for the $\alpha_4\beta_2$ and α_7 subtypes abolished the attenuating effect of RJR-2403 and PNU-282987 on acute ethanol ataxia by knocking down the expression of proteins for both subtypes. In the control animals pretreated similarly with missense oligonucleotides against $\alpha_4\beta_2$ and α_7 subtypes mRNA, the normal attenuating effect of RJR-2403 and PNU-282987 was observed. The results of antisense and missense experiments provided further confirmation that $\alpha_4\beta_2$ and α_7 subtypes play a role in the functional interaction of nicotine and ethanol.

This dissertation research also demonstrated that repeated ICB microinfusion of various doses of RJR-2403 and PNU-282987 on acute ethanol ataxia resulted in the development of cross-tolerance between nicotine and acute ethanol ataxia in a dose dependent manner. The optimal cross-tolerance was observed in 5-day nicotine/RJR-2403 ICB treatment group and it lasted for 48 hours. The dose dependency of the development of cross-tolerance indicated the absence of nicotine receptor desensitization up to 5-day treatment but significant nAChRs desensitization was observed following 7-day nicotine/RJR-2403 treatment. The peak of cross-tolerance was observed only after 1-day PNU-282987 treatment and lasted for 72 hours. The results of the chronic studies were in agreement with the acute experimental data and confirmed the involvement of $\alpha_4\beta_2$ and α_7 nAChR subtypes in the nicotine and ethanol interaction. Repeated pretreatment with the

$\alpha_4\beta_2$- and α_7- selective antagonists, DHβE and methyllycaconitine, respectively, blocked the development of the cross-tolerance indicating the mediating role of $\alpha_4\beta_2$- and α_7 subtypes. Despite several reports regarding distribution of nAChR subtypes, controversy still exists regarding whether $\alpha_4\beta_2$ and α_7 subtypes are present in cerebellum (Gotti and Clementi, 2004; Nashmi and Lester, 2006). We attempted to immunohistochemically determine the comparative cerebellar distribution of most predominant nAChR $\alpha_4\beta_2$ and α_7 subtypes (Paterson and Nordberg, 2000; Gotti and Clementi, 2004; Nashmi and Lester, 2006). We have previously shown that sodium nitroprusside (a NO donor), and isoliquiritin (an activator of guanyl cyclase) augmented nicotine's ability to counter ethanol ataxia whereas S-methylisothiourea (an inhibitor of inducible NO synthase), and ODQ (1H [1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one) (an inhibitor of guanyl cyclase) diminished nicotine's attenuating effect on ethanol ataxia suggesting participation of NO signaling. Consequently, the dissertation research measured cerebellar concentrations of NO following intracerebellar microinfusion of acute/repeated RJR-2403 and PNU-282987, $\alpha_4\beta_2$ and α_7 subtype agonists, respectively, in the presence or absence of acute alcohol. Information regarding the participation of specific nAChR subtypes in the alcohol – nicotine interaction defined from the proposed research may ultimately lead to the development of therapeutic agents (e.g. agonists and antagonists of nAChRs subtype(s)) which may permit: (i) to curb factors which drive smokers to seek alcohol kick or vice versa (antagonist drug of nAChRs subtype(s); and (ii) to attenuate the degree of motor impairment due to alcohol consumption (agonist drug of nAChRs subtype(s).

Nicotine History

Historical records show that the ancient tobacco plant “nicotinna tabacum” was used for more than 10000 years by Central, North and South Americans for ceremonial and religious purposes (Gately, 2001). Medicinally, the tobacco plant was known to heal wounds and suppress appetite. To exploit medicinal benefits, the plant was snuffed, chewed, smoked and drunk as a liquid brew (Gately, 2001; Kuper et al., 2002). During the 14-16th century, the tobacco plant was introduced globally followed by a booming era of cigar and cigarette smoking. Despite awareness of serious health risks associated with its use, smoking of tobacco, which is a highly addictive substance, continues to be wide spread. Addiction to tobacco is largely due to its principle alkaloid, nicotine. Along with nicotine, tobacco smoke contains 4,000 highly toxic and carcinogenic substances including, organic solvents, heavy metals, carbon monoxide etc (Haustein, 2003).

Currently, nicotine in the form of smoking is ranked as the most exorbitantly consumed substance of abuse. Statistically, about 30% of the American population (70-80 million) indulged in nicotine abuse in the form of smoking cigarette, cigars, pipes and chewing gums. In the US alone, tobacco consumption is responsible for nearly 400,000 deaths/year due to lung cancer, stroke, cardiovascular and respiratory diseases, with lung cancer being the third leading cause of preventable deaths (Mokdad et al., 2004)(Table 1).

Nicotinic Acetylcholine Receptors (nAChRs)

The physiological and pharmacological actions of nicotine are mediated by specific nicotinic acetylcholine receptors (nAChRs): the well characterized members of the superfamily of ligand gated ion channels. Intrinsically, nAChRs are pentameric ligand

TABLE 1: Annual causes of deaths in the United States (Mokdad et al., 2004)

Tobacco	**440,000**
Poor physical inactivity	365,000
Alcohol	85,000
Microbial agents	75,000
Toxic agents	55,000
Motor vehicle crashes	26,347
Adverse reaction to prescription drugs	32,000
All illicit drug use	17,0000

gated ion channels that are composed of different combinations of distinct subunits e.g. one of the eight α (α_2- α_9) and, of three β (β_2- β_4), each encoded by a different gene. The assembly of distinct α and β subunits constitutes, in various combinations, a number of homomeric and heteromeric nAChRs subtypes (Paterson & Nordberg, 2000; Nott and Levin et al. 2006) i.e. α_2- α_4 and β_2/ β_4 receptor subunits form a pentameric structures only in association with other subunits while α_7-α_9 can assemble together to form homomeric nAChRs subtypes of identical subunits. Examples of heteromeric receptors are $\alpha_2\beta_2$, $\alpha_2\beta_4$, $\alpha_3\beta_4$, $\alpha_3\beta_2$, $\alpha_4\beta_2$, $\alpha_2\beta_3$ and of homomeric subtypes are α_7, α_9. The pentameric subunits assemble around a central ion pore with an M2 region lining the central pore of the receptor (Paterson & Nordberg, 2000).

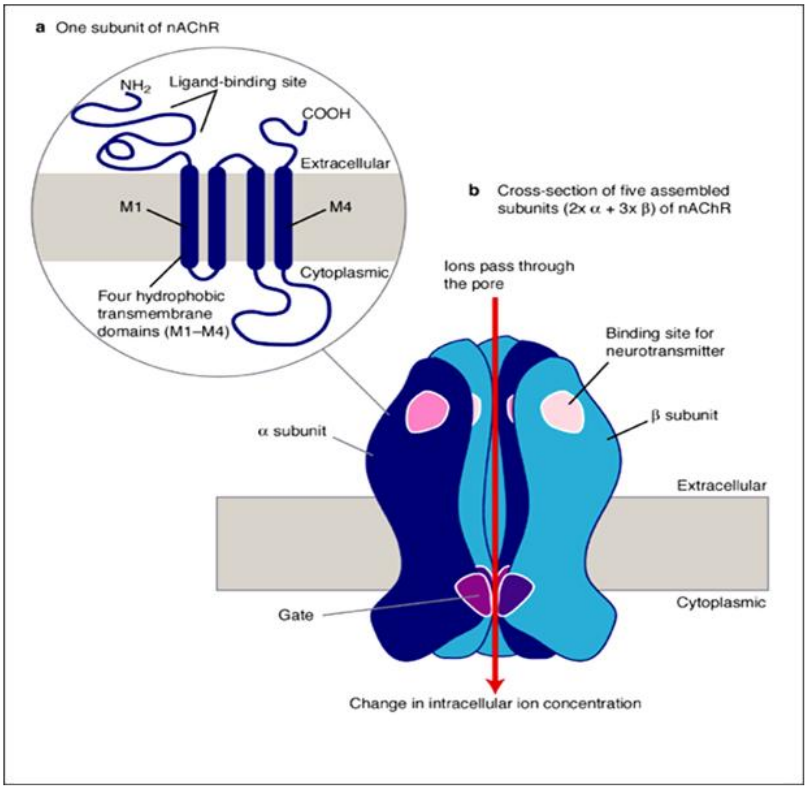
Not only both homomeric and heteromeric nAChR subtypes are distinct in terms of their pharmacological and physiological behavior but individual receptor subtypes in each category have their own unique characteristics. This signifies the contribution of each receptor subunit (α or β) in determining the overall properties of the receptor subtype (Papke et al., 1993; Wang et al, 1996). Ubiquitously distributed, neuronal nAChRs are differentiated by their kinetic properties, ligand binding characteristics, localization, subunit composition, pharmacological and functional properties and cation permeability. Of all the nAChR subtypes, those composed of $\alpha_4\beta_2$ and α_7 exhibit highest nicotine sensitivity with $ED_{50\%}$ values of 1-5μM and 40μM, respectively (Gotti et al. 1997).

Structure of Nicotinic Acetylcholine Receptors

The Topography of nAChRs as revealed by earlier crystallographic studies have identified the following components of individual subunits of the nAChRs :1) an extracellular hydrophilic amino terminus (-NH2-) of 200 amino acids bearing agonist

binding sites, 2) a compact hydrophobic domain consisting of four transmembrane (TM) spanning domains, M1-M4; 3) a selectivity filter which lies between the M3-M4 domains; and 4) an intracellular hydrophobic loop between domains M3-M4 that contains phosphorylation sites for serine/threonine kinases (Fig. 1) (Galzi & Changeux, 1995; Corringer et al., 2000). The homomeric nAChRs subtypes exhibit five acetylcholine binding sites , one on each α subunit at the interface between two adjoining α subunits, while heteromeric receptor subtypes with two α and 3 β subunits have only two ligand binding sites at the interface between α and β subunits. As these acetylcholine binding sites have functionally cooperative interaction, it requires five and two ligand molecules to activate homomeric and heteromeric nAChRs, respectively (Changeux & Edelstein, 1998; Corringer et al., 2002). Besides, nAChRs bear dihydropyridine, steroid and allosteric activator and inhibitor sites which offer enormous opportunities for pharmacological manipulation of the nAChRs. Among the allosteric activators are tacrine, benzoquinonium, acetylcholinesterase inhibitors such as physostigmine and neostigmine (Svensson & Nordberg, 1996; Schrattenholz et al., 1996). The allosteric binding site in $\alpha_4\beta_2$ is presumably located on α-subunit. These compounds act to enhance channel opening frequency (Pereira et al., 1993) and ion conductance as well as to upregulate binding sites (Svensson & Nordberg, 1996). The allosteric inhibitors include, ethanol, phencyclidine, MK-801, chlorpromazine, local anesthetics and barbiturates. The binding of allosteric inhibitors to their distinct sites (high affinity, low affinity sites) does not prevent ligand binding to acetylcholine sites but reduces the open time of channel (Nagata et al., 1996). Besides, nAChRs bear dihydropyridine, steroid and allosteric activator and inhibitor sites

FIGURE 1: General structure of an ionotropic neuronal nicotinic acetylcholine receptor (nAChR) (Bate & Gardiner, 1999)


a One subunit of nAChR
NH2
Ligand-binding site
COOH
Extracellular
M1
M4
Cytoplasmic
Four hydrophobic transmembrane domains (M1–M4)
b Cross-section of five assembled subunits (2x α + 3x β) of nAChR
Ions pass through the pore
Binding site for neurotransmitter
α subunit
β subunit
Extracellular
Cytoplasmic
Gate
Change in intracellular ion concentration

which offer enormous opportunities for pharmacological manipulation of the nAChRs. Among the allosteric activators are tacrine, benzoquinonium, acetylcholinesterase inhibitors such as physostigmine and neostigmine (Svensson & Nordberg, 1996; Schrattenholz et al., 1996). The allosteric binding site in $\alpha_4\beta_2$ is presumably located on α-subunit. These compounds act to enhance channel opening frequency (Pereira et al., 1993) and ion conductance as well as to upregulate binding sites (Svensson & Nordberg, 1996). The allosteric inhibitors include, ethanol, phencyclidine, MK-801, chlorpromazine, local anesthetics and barbiturates. The binding of allosteric inhibitors to their distinct sites (high affinity, low affinity sites) does not prevent ligand binding to acetylcholine sites but reduces the open time of channel (Nagata et al., 1996).

Distribution of Nicotinic Acetylcholine Receptor Subtypes in the CNS

As yet, the distribution of nAChRs has been thoroughly explored in invertebrates, primates, rodents and humans in the and peripheral nervous system as well as in non-neuronal sites including skin, lung, immune cells and the cardiovascular system (Paterson & Nordberg, 2000; Gotti & Clementi, 2004; Nashmi & Lester, 2006). The current knowledge of cellular/subcellular distribution of nAChRs is based on immunohistochemistry, immunoprecipitation, immunoblotting and radioligand binding techniques. The relative selectivity or specificity of certain ligands or antibodies for one nAChR subtype over other subtypes forms the basis for the identification of different nAChRs subtypes. For instance, [^{3}H]-cytisine displays the highest affinity for the $\alpha_4\beta_2$ subtype and [^{3}H]-methyllycaconitine or [^{125}I]-α-BTX for α_7 subtypes and so are used for labeling of their respective subtypes (Ward et al., 1990). [^{3}H]-nicotine and [^{3}H]-epibatidine are rather nonselective ligands and bind $\alpha_4\beta_2$, and other subtypes, notably $\alpha_3\beta_2$, $\alpha_3\beta_4$

(Flores et al., 1997; Zoli et al., 1998) in low doses and α_7 at high doses (Ward et al., 1990). Since $\alpha_4\beta_2$ and α_7 subtypes bind nicotine with high (nM) and low affinity (µM) respectively, these subtypes are termed as high and low affinity subtypes.

In humans, [^{3}H]-nicotine binding sites were found distributed densely in the thalamus and caudate-putamen, moderately in the cortex (occipital, fronto-parietal), sparsely in the temporal cortex, hippocampus and cerebellum while no binding sites were shown in the hypothalamus, pineal gland and medial habenula. Conversely, [^{125}I]-α-BTX binding was rich in hippocampus, nucleus reticularis, pontine nucleus, area broca and inferior olivary nucleus, moderate in the hypothalamus, pons and medulla and low in the cortex and cerebellum (Gotti and Clementi, 2004). Thus, low but detectable levels of [^{3}H]-nicotine and [^{125}I]-α-BTX binding was found in the human cerebellum. The cerebellar granular layer contained more [^{3}H]-nicotine bindings sites than the molecular layer (Court et al., 2000); while the molecular layer has more [^{125}I]-α-BTX binding sites than the granular layer (Aubert et al.,1992). Similar observations were made with immunohistochemical studies that demonstrated that Purkinje cells express a high density of α_4, α_7 and moderate to weak levels of α_3, α_4, α_6, β_2, β_4 while granule cells contained α_4, β_2, β_4 but not α_3 in human cerebellum (Graham et al., 2002).

In rodents, the generalized profile of nAChR distribution is not markedly different from those of human and primates, though some species related discrepancies exist. [^{3}H]-nicotine binding sites are highly abundant in the thalamus, cerebral cortex (layer III/IV), interpeduncular nucleus and superior colliculus (Clarke et al., 1985); moderately abundant in the parietal cortex, cingulate cortex, subiculum, substantia nigra, optic nerve dorsal raphe, laterodorsal tegmental nucleus and retrospenial cortex; and low in the medial

septum, nucleus accumbens, caudate putamen, hippocampus and olfactory tubercle (Marks et al., 1992). α-BTX-binding was concentrated in the hippocampus, hypothalamus and cortical layers I/VI (Clarke et al., 1985). However, the distribution of nAChR subtypes in the cerebellum has been controversial. While Decker et al. (1999) have found a moderate expression of [^{3}H]-nicotine binding sites and nil for [^{125}I]-α-BTX binding sites (see Table 2), Nashmi and Lester (2006) did not detect any nAChR binding in rat cerebellum.

However, some recent studies, investigating cellular and subcellular details, do support the existence of nAChRs in the cerebellum (Nakayama et al., 1997,1998). For example, a high α_4 transcript level had been detected in the Purkinje and granule layers while less intense signal was noted in the molecular layer (Nakayama et al., 1998). Immunohistochemica studies, using α_4 selective monoclonal antibodies in adult Wistar rats have also revealed a similar pattern of distribution of α_4 receptor protein in the molecular, Purkinje and granule layers, with the strongest signal in the Purkinje cell layer. The subcellular regions immunostained for α_4 subunits in Purkinje cells include the soma, plasma membrane, rough endoplasmic reticulum, perikarya, axo-somatic synapse and extrasynaptic sites as well as the granule cell somatic membranes (Nakayama et al., 1998). Other studies also support the existence of α_4, β_2 immunoreactivity in Purkinje, granule and deep cerebellar nuclei with α_7 subtype more prominently expressed in Purkinje than the granule cells (Hill et al., 1987; Swanson et al., 1987; Wada et al., 1989; Caruncho et al., 1997). In granule cells, α_7 expression was not detected in the soma but rather was found only in dendrites (Caruncho et al., 1997).

TABLE 2: Autoradiographic and in situ hybridization analysis of the distribution of nicotinic binding sites and mRNA in rat brain (Adapted from Decker et al., 1999)

	Ligand Binding		Subunit mRNA Distribution								
Region	**Nicotine**	**α-Bgt**	**α2**	**α3**	**α4**	**α5**	**α6**	**α7**	**β2**	**β3**	**β4**
Forebrain											
Cortex	+++	++	-	+	++	+	-	+	++	-	(+)
Hippocampus	+	+++	(+)	+	+	+	-	+++	++	-	+
Thalamus	+++	-	-	+	+++	-	+	-	++	++	+++
Hypothalamus	+	+++	-	-	+	-	-	-	+	-	-
Striatum	++	-	-	(+)	-	-	-	-	+	-	(+)
Septum	++	+	-	-	+	-	-	+	+	-	(+)
Brainstem											
Motor Nuclei	++	+	-	++	+	+	-	-	++	-	+
SN	++	-	-	++	++	++	+++	-	++	+++	(+)
LC	++	++	-	++	-	-	+++	-	++	+++	++
IPN	+++	++	++	+++	+	++	+	(+)	++	+	++
Cerebellum	++	(+)	-	+	-	+	-	-	+	+	+

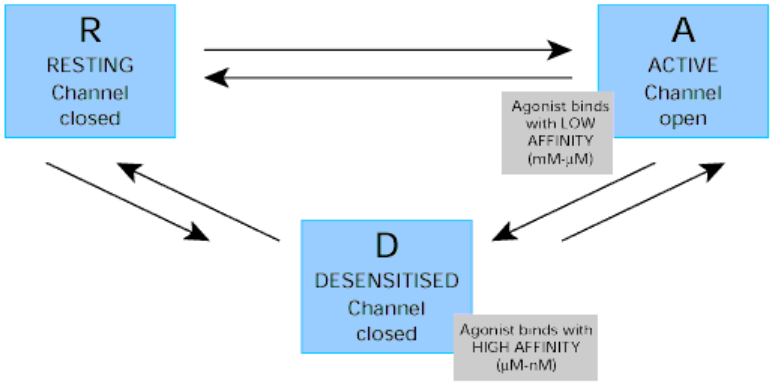
[b]Key: -, not detectable; (+), very weak signal; +, weak signal; ++, moderate signal; +++, strong signal; IPN, interpeduncular nucleus; LC, locus coeruleus; SN, substantia nigra.

The expression of many nAChR subunits is regulated during developmental stages. For example, α_4, α_3 and β_4 mRNA transcript is highly expressed during postnatal days P1-P14 and then declines to adult level (De Filippi, et al., 2001). Similarly, an α_3 and β_4 gene transcript is seen during the embryonic phase but not in P5 (Zoli et al., 1995; Winzer-Serhan & Leslie, 1997). The β_4 mRNA level also fluctuates during the postnatal period; a weak signal at P5; moderately high after P11 and remain consistent until adulthood. The expression of β_2 mRNA has been reported to be consistent during development. Both α-BTX labeling and mRNA levels were low in the cerebellar cortex during development (Zhang et al., 1998b). However, the level rises and peaks at P14 followed by a continued decline until it reaches the adult level: about ten times less than P1 (De Filippi et al., 2005). It was also demonstrated by electrophysiological studies that α_7 subtypes are localized to the mossy fiber-granule cell synapse during the early developmental period (De Filipi et al., 2001). The disparity in receptor localization, reported by different investigators, may be explained by variation in experimental conditions, tissue selection, choice of ligands/antibodies, incubation time, species or strain differences.

Desensitization of Nicotinic Acetylcholine Receptors and its Functional Significance

Nicotinic receptors display three kinetically different conformational states: 1) resting state that has the highest affinity for the antagonists); 2) an activated state in which the channels are active and remain open for a brief time period (i.e. µsec to msec) but retain low affinity for the ligands; 3) desensitized state in which the channels are resistant to opening despite high agonist affinity (pM-nM) (Fig. 3)(Galazi and Changeux, 1995).

FIGURE 2: Schematic models for three transient states of nicotinic acetylcholine receptors (Balfour et al., 2000).


R
RESTING
Channel
closed
A
ACTIVE
Channel
open
Agonist binds
with LOW
AFFINITY
(mM-μM)
D
DESENSITISED
Channel
closed
Agonist binds with
HIGH AFFINITY
(μM-nM)

The transition among these different conformational states is regulated by triggering PKA and/or PKC-dependent phosphorylation of nAChR. Both PKC and Ca^{2+} seem to regulate recovery from the desensitization phase as either replacement of external Ca^{2+} with Ba^{2+} or inhibition of PKC delayed recovery from nicotine-induced desensitization in *Xenopus laevis oocytes* (Fenster et al., 1999). There is also evidence that inhibition of tyrosine kinase increases the expression of and related response by the α_7 subtype (Cho et al., 2005).

Various nAChR ligands exhibit selective affinity to a particular receptor state and can stabilize the receptor conformation in that particular state (e.g. the agonists will stabilize the open state from which receptors can transform into desensitized state), whereas antagonists can stabilize the closed state. However, long exposure to the agonist can transition the receptor into a state of permanent inactivation (Karlin, 1993; McGehee and Role, 1995). Desensitization of nAChRs represents a conservatory response to protect against cellular damage and synaptic changes following excessive receptor stimulation and Ca^{2+} build up. The rate, extent and duration of desensitization varies with agonists, exposure time, and receptor subtype involved (i.e. desensitization is faster for the α_7 subtype than $\alpha_3\beta_2$ and $\alpha_2\beta_2$) while the $\alpha_4\beta_2$ desensitizes more slowly than the mentioned subtypes (Alkondon and Albuquerque, 1993; Vibat et al., 1995). Overall, the most predominant α4β2/α7 subtypes exhibit the highest sensitivity to agonist-induced desensitization and upregulation (Hsu et al., 1996). The stoichiometric subtype composition of nAChRs is also a factor for differential rates of receptor desensitization and upregulation among various nAChR subtypes. By far, $2\alpha_4$:$3\beta_2$ composition has a higher

rate of desensitization and upregulation than $4\alpha_4$:$1\beta_2$ and $1\alpha_4$:$4\beta_2$ (Lopez-Hernandez et al., 2004). The desensitization phenomenon has been attributed to nicotine addiction (Buisson and Bertrand, 2002; Gentry and Lukas, 2002), tolerance (Robinson et al., 2007) and nAChRs upregulation in humans (Breese et al., 1997), in rodents (Zhang et al., 1994; Nguyen et al., 2004) and in various cell lines (e.g. human epithelial cells [SH-EP1]), cultured enteric neurons, human embryonic kidney cells (HEK-293), chinese hamster ovary (CHO-K1) and pheochromocytoma cells (PC12) (Fenster et al., 1999; Buisson & Bertrand, 2002; Decker and Galligan, 2009).

Smoking a single cigarette can elevate brain nicotine levels to 220-330nM (Henningfield et al., 1993), while nicotine can desensitize the $\alpha_4\beta_2$ nAChR subtype at a concentration of 10nM as measured by decay of peak current (Paradiso et al., 2003). With several episodes of smoking, smokers can produce basal nicotine levels that are far higher than the concentration required to desensitize a significant proportion of nAChRs receptors in brain. This explains why smokers are tolerant to several actions of nicotine and that they have high nAChRs in selected brain regions such as nucleus accumbens, superior colliculus and hippocampus (Breese et al., 1997; Nashmi et al., 2007). Prolonged exposure to nicotine may inactivate α_4/α_7 subtype nAChRs rather than produce desensitization them which may account for the significant increase in density of these subtypes in a smoker's brain (Peng et al., 1994; Olale et al., 1997).

Long-term nicotine exposure upregulates both $\alpha_4\beta_2$ and α_7 binding sites in a variety of subjects like rodents (Marks et al., 1992; Nguyen et al., 2004), human (postmortem) brain (Breese et al., 1997) and *in vitro* cells including, HEK 293, MIO, oocytes (Gopalakrishnan et al., 1996; Eilers et al., 1997; Molinari et al., 1998). Other agonists

(epbatidine, anabaseine, 1,1-dimethy 4-phenylpiperazinium) also produce an increase in $\alpha_4\beta_2$ and α_7 binding sites (Eilers et al., 1997). Interestingly, upregulation can only be triggered by ligand interaction with cell surface receptors as tetramethylammonium, a membrane-impermeant ligand that potently increases receptor number similar to nicotine while [^{3}H]-epibatidine did not upregulate cell surface receptors as its binding sites lie intracellularly. On the other hand, the response of antagonists has been somewhat controversial since DHβE, methyllycaconitine, tubocurarine, hexamethonium did not alter receptor expression when tested in M10 cells and SH-SY5Y neuroblastoma cells (Eilers et al., 1997; Riganti et al., 2005) while a relatively new antagonist, CC4, enhances both [^{3}H]-epibatidine or ^{121}I-α-bungarotoxin binding sites on SH-SY5Y (Riganti et al., 2005). Epibatidine, a partial agonist at $\alpha_4\beta_2$ subtype containing receptors upregulates the receptors at sub-effective concentrations without activating the receptors (Buisson et al., 2002).

Given the fact that a long-term increase in nicotine exposure is accompanied by two related phenomena of desensitization and upregulation, it was proposed that desensitization may provide a trigger for receptor upregulation (Mudo et al., 2007). However, Kishi and Steinbach (2006) clearly demonstrated that these two effects are distinct phenomena as mutation of amino acid residues in nAChRs that were required for desensitization did not affect receptor upregulation while mutation of the amino acids in the binding site prevented receptor upregulation. In addition, the concentration requirement for receptor desensitization varied greatly from that necessary for receptor upregulation.

Nicotinic Acetylcholine Receptors and Cellular Changes

The nAChRs are mainly associated with the conductance of fast synaptic transmission. Low concentrations of nicotine (nM) can adequately alter the electrophysiological environment of neurons by promoting the cationic (Na^+, K^+) influx and burst opening of the voltage-gated ion channels. During this initial depolarization phase, Ca^{2+} influx is paramount in amplifying the initial signal by maintaining a continual supply of Ca^{2+} from extracellular and intracellular storage sites. The sustained Ca^{2+} signal is implicated in neurotransmitter release (McGehee & Role, 1995; MacDermott et al., 1999), and long-term adaptive changes (Dajas-Bailador et al., 2002; Dickinson et al., 2007). In this context, synaptic plasticity including, long-term potentiation (LTP) is paramount in neuronal functions such as learning, memory and addiction (McKay et al., 2007). The cellular signaling molecules involved in the cognitive function includes, the cAMP response element binding protein (CREB) and ERK/MAPK. Other downstream cellular events that account for nicotine's action and are relevant to nicotine receptor activation include, modulation of cGMP-NO signaling, and PKC activity (Dajas-Bailador et al., 2002). After chronic treatment, nicotine upregulates the mRNA transcripts of tyrosine hydroxylase (TH) in *in vivo* as well as *in vitro* chromaffin cells. The induction of TH mRNA is a consequence of sequential events that involve sustained high Ca^{2+} levels and activation of ERK/MAPK (Haycock, 1993). Chronic nicotine exposure also induces many other immediate early genes (e.g. cfos, c-Jun), transcription factors, protein processing factors, RNA binding and plasma binding proteins (Konu et al., 2001; Hu et al., 2002; Ho et al., 2004). Since glutamate has been regarded a major neurochemical mediator for nicotine's action, a detailed review of the nicotine-glutamate system is provided in the following section.

Functional Relationship between Nicotine, Glutamate and Nitric Oxide

There is sufficient evidence in the literature to support the idea that nicotine modulates the glutamatergic system. Nicotine increases glutamate release in rat nucleus accumbens after acute as well as chronic treatment (Lallemand et al., 2006). The α_7 nAChR subtype appears to exert a more modulatory influence on nicotine-glutamate related functions (e.g. LTP, transmitter release) than any other nAChR subtype as was shown in a study where nicotine and a selective α_7 agonist, choline; augmented 4-aminopyridine evoked glutamate release in rat prefrontal cortex nerve terminals through Ca^{2+}-calmodulin pathways (Girod et al., 2000; Reno et al., 2004; McKay et al., 2007). However, in frontal cortex, the $\alpha_4\beta_2$ subtype regulates the release of the excitatory transmitter [^{3}H]-D-aspartate (Rousseau et al., 2005). *In vitro* data has also shown that nicotine enhanced the excitatory current at postsynaptic NMDA receptors in brain slices containing the amygdala (Kenny et al., 2009). Another evidence of cross-talk between nicotine and the glutamate system comes from the evidence that intra-NTS microinjection of nicotine elevates norepinephrine levels in PVN and AMYG and that this response can be reduced by pretreatment with the NMDA receptor antagonist (DL-2-amino-5-phosphonopentanoic acid) but not the AMPA receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Zhao et al., 2007). Nicotine-induced dopamine release in nucleus accumbens was markedly attenuated by NMDA and NOS inhibitors, MK-801 and L-NNA (N-nitro-L-arginine) (Hong et al., 2006).

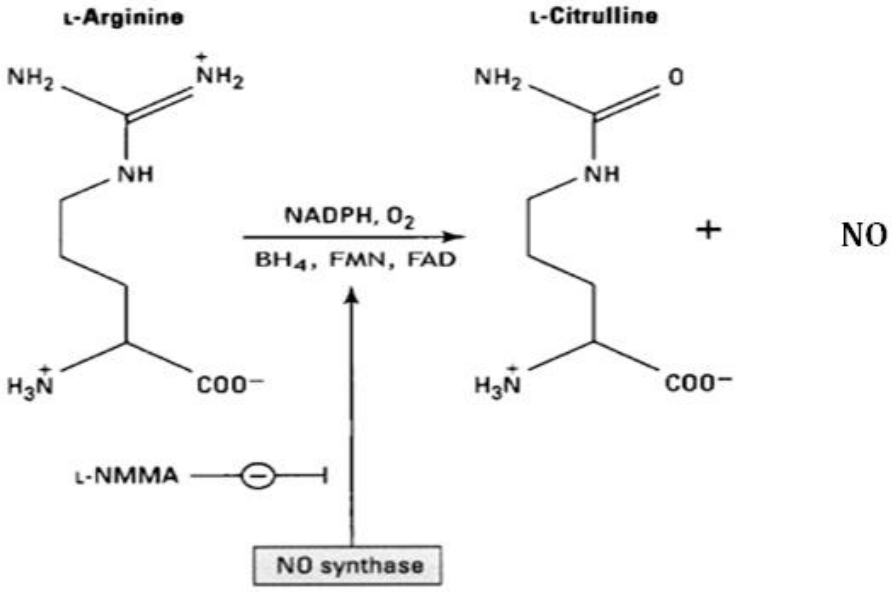
The glutamate system may also play an important role in addiction to nicotine since the NMDA receptor antagonist, LY 235959, reduced nicotine self-administration without interfering with normal food intake (Kenny et al., 2009). Also, nicotine's effect on cognition

depends upon glutamate-NO signaling because the NMDA antagonist, MK-801 and nNOS knockout mice display significant impairment in learning and memory performances. (Levin et al., 1998; Wultsch et al., 2007).

Nicotine-induced glutamate release leads to downstream activation of NO-cGMP signaling in several brain regions such as the cortex, hippocampus and cerebellum through an NMDA-receptor mediated mechanism since MK-801 and nitric oxide synthase (NOS) inhibitors abolish nicotine-induced NO release (Damaj et al., 1999; Pogun et al., 2000). Nicotinic receptor activation also facilitates cationic (Na^+, K^+, Ca^{2+}) influx leading to elevated intracellular Ca^{2+} levels which when combined with calmodulin stimulates NOS to liberate NO (Ma et al., 2008). Nitric oxide is a biochemical molecule synthesized by NOS from the precursor, l-citrulline (Fig. 3) (Stuehr & Griffith, 1992; Morris, 2005). Nitric oxide synthase can also serve as an important neurochemical target site whereupon nicotine-glutamate alters neuronal function (Cooper and Magwere, 2008; Ward et al., 2008).

Nicotine exposure has also been shown to upregulate NMDA receptors in the amygdala and the VTA (Kenny et al., 2009). We have also demonstrated that intracerebellar microinfusion of nicotine attenuates ethanol ataxia in CD-1 mice through glutamatergic signaling downstream of nAChR activation since intracerebellar microinfusion of NMDA or AMPA exacerbated while MK-801 and NBQX, selective receptor antagonists at NMDA and AMPA receptors, respectively diminished the attenuating action of nicotine on ethanol ataxia (Al-Rejaie and Dar, 2006a). Since NO/cGMP pathway is triggered subsequently to NMDA receptor activation, it is tempting to speculate that NO signaling may play a role in the mediation of nicotine's effects. Additionally, it was found that pretreatment with SNP (sodium nitroprusside, a NO donor)

FIGURE 3: Biochemical synthesis of nitric oxide from precursor L-arginine (Ghalayini et al., 2004).


L-Arginine
L-Citrulline
NADPH, O2
BH4, FMN, FAD
+
NO
L-NMMA
NO synthase

and isoliquiritigenin, a guanylyl cyclase activator also markedly elevated nicotine-induced attenuation of ethanol ataxia. On the other hand, pretreatment with SMT (an inhibitor of iNOS) and ODQ (an inhibitor of GC), diminished the nicotine-induced antagonism of ethanol ataxia (Al-Rejaie and Dar, 200a). Repeated intracerebellar nicotine treatment resulted in reversible cross-tolerance between nicotine and ethanol ataxia (Al-Rejaie and Dar, 2006b, c). Again cerebellar NO production downstream of nAChRs contributed to the development of cross-tolerance (Al-Rejaie and Dar, 2006b, c).

Outside the CNS, NO also mediates several functions of nicotine (Ayajiki et al., 1997; Zhang et al., 1998a). In the vascular system, nicotine-induced relaxation occurs through NO release (Ayajiki et al., 1997). Nicotine generates NO from NANC nerves in gastrointestinal smooth muscles to relax the sphincter of Oddi (Tanobe et al., 1995). Guanylyl cyclase is a major cellular target for NO, which regulates basal extracellular levels of cGMP in several brain regions including the cerebellum (Laitinen et al., 1994). It has been shown that NOS inhibition by N-nitro-L-arginine methyl ester (L-NAME) reduced cGMP production by 74% in the cerebellum whilst NO donor, SNP, augmented cGMP levels by 90-fold (Laitinen et al., 1994). According to another study hippocampal extracellular cGMP was measured following systemic nicotine in conscious mice. The cGMP levels were raised after nicotine treatment and declined after localized injection of NOS inhibitor, N(G)-nitro-L-arginine (L-NOARG) and GC inhibitor, 1H-[1,2,4] oxadiazolo [4,3-A] quinoxalin-1-one (ODQ) (Fedele et al., 1998).

Pharmacology of Nicotine

Nicotine is one of the most highly addictive substances known to mankind. The pleasurable effects of nicotine appear in the form of cognitive enhancement (learning,

memory, and judgment), enhanced alertness, visual attention (Jones et al., 1992), increased motor skills, anxiolytic actions, locomotor modulation and antinociception (Hindmarch et al., 1990; Jones et al., 1992). The self-rewarding effects of nicotine are due to DA release from the nucleus accumbens in the mesolimbic system (Clarke, 1990; Balfour et al., 1998) as shown in numerous *in vivo* and *in vitro* studies (Tizabi et al., 2002; Singer et al., 2004; Rodvelt et al., 2006), while nAChRs antagonists could abolish nicotine-induced DA release and systemic nicotine self-administration (Neugebauer et al., 2006; Dwoskin et al., 2008).

Nicotine's effects on cognition improvement are very well established. More significant effects of nicotine or related nicotinic receptor agonists are observed on working memory than reference memory (Levin, 2002; Levin and Simon, 1998). Conversely, the nicotinic receptor antagonist, mecamylamine, not only prevents the nicotine-induced improvement in memory but also disrupts working memory performances (Levin and Simon, 1998). These data clearly delineate the essential involvement of nAChR system in regulation of memory function. Anatomical localization of $\alpha_4\beta_2$ and α_7 subtypes in memory processing areas and the fact that selective antagonists at these subtypes, DHβE and methyllycaconitine impair working memory functions indicate that both subtypes actively participate in maintaining normal memory functions (Addy et al., 2003).

Nicotine has a profound impact on regulating the release of a variety of neurotransmitters including, norepinephrine, dopamine, 5HT, acetylcholine, glutamate, GABA (McGehee and Role, 1995; Picciotto et al., 1998; MacDermott et al., 1999), CGRP, substance P (Sastry, 1995) and growth factors such as BDNFs or FGFs which

play a neuroprotective role mostly through presynaptic or preterminal nAChRs in the CNS (French et al. 1999). Conversely, exogenously applied nAChRs antagonists can diminish neurotransmitter release.

Unlike some recreational drugs like alcohol, heroin, cocaine and marijuana, nicotine does not have neurotoxic actions, rather it exhibits neuroprotective functions by enhancing the expression of neurotropic factors and nerve growth factor receptors as well by increasing the release of growth factors in several brain areas such as the cerebral cortex, hippocampus, striatum and midbrain (Belluardo et al., 2000) as well as in chromaffin cells in culture (Stachowiak et al., 1994). In hippocampal slices, nicotine protects against acute NMDA-induced neurotoxicity through the phosphatidylinositol 3-kinase and ERK/MAPK pathways (Ferchmin et al., 2003). The neuroprotective actions of nAChRs form the basis for therapeutic prospects for nAChRs agonists in the treatment of neurodegenerative diseases (i.e. Alzheimer's and dementia). The most notable underlying mechanism for antineurotoxic actions is Janus kinases 2, PI3K phosphorylation of Akt (PKB) and upregulation of the anti-apoptotic marker Bcl-2 (Ferchmin et al., 2003; Mai et al., 2003) while prevention of glutamate toxicity involves the ERK/MAPKinases (Ferchmin et al., 2003). Chronic exposure to nicotine or its related agonists desensitize nAChRs, which also participate in providing neuroprotection by shutting off excessive nAChR dependent intracellular Ca^{2+} influx that evokes neurodegenerative processes (Dani, 2001).

Role of $\alpha_4\beta_2$ subtypes in Nicotine Action

Among the diverse variety of nAChRs subtypes, $\alpha_4\beta_2$ has received the most attention due to its predominant distribution and its important role in mediating wide range of nicotine actions (Damaj et al., 1995a; Picciotto et al., 1998; Marubio et al., 1999; Perry,

et al., 2002; Wùllner et al., 2008). For example, the $\alpha_4\beta_2$ subtype is involved in nicotine-induced analgesia, dopamine release, hyperthermia and locomotor stimulation (Damaj et al., 1995a; Cohen et al., 2003). Within $\alpha_4\beta_2$, it is the β_2 subunit of nAChRs which participates in nicotine's actions such as analgesia, enhanced nicotine craving and cognition (Picciotto et al., 1998; Marubio et al., 1999). Studies in transgenic mice have shown that deletion of the β_2 subunit abolishes nicotine's self-administrative behavior, indicating the potential role of the β_2 subunit in the addictive properties of nicotine (Piccioto et al. 1998).

The nAChR $\alpha_4\beta_2$ subtype is known to mediate the cognitive enhancing effects of nicotine. The selective $\alpha_4\beta_2$ agonist SIB-1508Y and the nonselective $\alpha_4\beta_2$ agonist, epibatidine demonstrate improved performance in learning and memory related tasks in various animal models (Vernier et a., 1999) while the $\alpha_4\beta_2$ selective antagonist, DHβE (30nmol), produces memory deficits in mice demonstrating the role of the β_2 containing subtype in cognition (Curzon et al., 1996). Additional support for this idea was provided by β_2 knockout mice that were devoid of any nicotine –evoked memory improvement (Picciotto et al., 1998).

Nicotinic receptors also promotes the release of neurotransmitters including, acetylcholine, GABA, dopamine, glutamate as well as growth factor release through $\alpha_4\beta_2$ receptor subtype activation and thereby imparts neuroprotective effects (Beani et al., 1985; McGehee and Role, 1995; Pontieri et al., 1996; Marshall et al., 1997; Kihara et al., 1998) since pretreatment with DHβE blocks this neuroprotective action of nicotine (Kihara et al., 1998). Though α_7 containing receptors are regarded equally important in this regard**.** We have shown that nicotine-induced attenuation of ethanol ataxia is primarily mediated by

cerebellar $\alpha_4\beta_2$ in mice based on the following observations: 1) the $\alpha_4\beta_2$ selective drug, (E)-N-Methyl-4-(3-pyridinyl)-3-buten-1-amine (RJR-2403) reverts ethanol-induced ataxia in a dose dependent manner; 2) the $\alpha_4\beta_2$-selective antagonist, dihydro-β-erythroidine hydrobromide (DHβE), virtually abolished nicotine- or RJR-2403-induced attenuation of ethanol ataxia and; 3) α_4 antisense treatment abolished RJR-2403's effect on ethanol ataxia (Taslim et al., 2008).

Role of α_7 subtypes in Nicotine's Action

Given the highly dense population of α_7 receptors in the hippocampus; an area recognized for processing learning and memory, the α_7 subtype remains the primary subtype implicated in cognitive functions. While it has been established that both α4β2 and α7 signaling regulate cognitive functions and that their deficit impaired learning and memory acquisition (Addy et al., 2003; Nott and Levin, 2006), it is α7 activation that is essential for both reference as well as working memory, in contrast to α4β2 which participates in working memory only (Chan et al., 2007). Besides, a deficit of nAChRs, in particularly the α_7 population in hippocampus (Nott and Levin, 2006), amygdala and frontal cortex (Addy et al., 2003), is correlated with cognitive impairment seen in Alzheimer and schizophrenic patients.

The second major role of α_7 subtype is to facilitate acetylcholine and glutamate release that was prevented by α_7 receptor antagonist, α-bungarotoxin and α_7 antisense oligo targeted to α_7 subunit (McGehee and Role, 1995; Girod et al., 2000). On the other hand, postsynaptically, α_7 subtypes are co-localized with $GABA_A$ receptors on rodent hippocampal interneurons and chick ciliary ganglion where their stimulation dampens GABA current. Thus, it appears that α_7 signaling promotes excitatory transmission by

releasing glutamate and acetylcholine and by suppressing GABA release. Another intriguing role of α_7 subtype has been mentioned in relation with its involvement in cannabinoid abuse (Picciotto, 2003). Both $\alpha_4\beta_2$ (Picciotto, 2003; Dani and Bertrand, 2007) and α_7 (Rassoulpour et al., 2005; Dani and Bertrand, 2007) subtypes are located on VTA (Dani et al., 2001), where they regulate dopamine release and mediate rewarding effects of nicotine. However, only the α_7 subtype antagonist, methylylcaconitine when given systemically antagonized THC-induced self-discriminative effects and dopamine release and CB1 agonist, WIN 55, 212-[R] induced self-administration (Solinas et al., 2007). The α_7 subtype is also expressed in early developmental stages and is essential in neuronal growth (Levin et al., 1999). A brief summary of functional role of other nAChRs is provided in Table 3.

TABLE 3: Summary of the functional role of nAChRs

Nicotinic receptor subtypes	Functions	References
α_2	Excite inhibitory interneuron in pyramidal cells, nicotine withdrawal	Salas et al., 2009; Jia et al., 2009
α_3	General growth and survival	Xu et al., 1999
α_4	Neuroprotection, locomotion, antinociception	Marubio et al., 1999; Ryan et al., 2001; Ross e al., 2000
α_5	Ganglionic transmission, nicotine-induced seizures and locomotor activity, nicotine withdrawal	Wang e et al., 2002; Salas et al., 2003, 2005
α_6	Dopamine overflow, locomotion	Larsson and Engel, 2004
α_7	Cognition, neuroprotection, neurotransmitter release, sympathetic response, nicotine-induced seizures	Alkondon et al., 1997; Francesc hini et al., 2000; Bianchi et al., 2000; Laudenbach et al., 2002; Broide et al., 2002; Reno et al., 2004.
α_8	Found in chick nervous system only to form both homo- and heteromeric channels	Gotti et al., 1994
α_9/ α_{10}	Expressed extraneuronally, exhibit mixed nicotinic-muscarinic profile; also complex with α10 subunit to form functional channels; required for the development of synaptic connection in cochlear hair cells, nociception.	Elgoyhen et al., 1994; McIntosh et al., 2009
β_2	Cognition, neuroprotection, regulation of REM sleep, development of visual system, nicotine addiction and reward	Piciotto et al., 1998; Levin, 2002; laudenbach et al., 2002; Picciotto & Corrigal, 2002
β_3	Locomotion, dopamine release	Cui et al., 2003
β_4	Ganglionic transmission, ileal and bladder contractile response, nicotine-induced seizures	Wang et al.,2003

Ethanol: a Social Drink or a Drug of Abuse

Ethanol is among the oldest and most widely consumed substances of intoxication. The history of ethanol abuse dates back to Paleolithic age, about 8000 B.C. Unlike nicotine, no specific target site is attributed to ethanol's action. Yet, the broad spectrum of ethanol's action owes to its dynamic nature to interact and modulate a multitude of molecular and cellular targets including, $GABA_A$, NMDA, nAChRs, 5HT receptors (Harris, 1999), adenosine receptors (Dar et al., 1983; 1990) and PKC/PKA etc (Zhang et al., 2005; Newton and Messing, 2006). Ethanol's actions on the CNS are generally well correlated with blood alcohol concentration and are dependent on the site of action. The action of ethanol can range from anxiolytic, relaxation, sedation, impaired cognition, slurred speech and disinhibitory behavior at moderately low doses to respiratory depression, coma, and death at high doses. A hallmark of ethanol's intoxication is profound ataxia which is known to be produced by the modulation of excitatory-inhibitory neuronal activity by ethanol in cerebellum. It is therefore not surprising that ethanol drinking is responsible for a significant number of traffic accident-related fatalities (Caputo et al., 2007). Individuals exhibiting low sensitivity to motor impairing action of ethanol are more likely to develop alcoholism (Schuckit et al., 1994). Therefore, understanding the mechanism of action of ethanol in the cerebellum is an area of continuing investigation.

Suggested Signaling Targets for Ethanol

Owing to its high lipid solubility and small molecular size, ethanol traverses virtually every cell in the body and interacts with a myriad of targets to execute its action. Ethanol does not have a specific target or receptor site to act on and render its effects. Hence, ethanol utilizes a host of sites: potential targets of other endogenous or exogenous ligands

to deliver its function. Ethanol is considered a heterotropic ligand which binds a distinct site on the receptor spared by the specific ligand for that receptor (Li et al., 1998; Harris et al., 1999). Given below is a brief overview of cellular/molecular entities that are targeted by ethanol. Ligand gated ion channels are integral target sites that respond to ethanol at concentrations ranging from 30-100 mM. Among the most notable ligand gated ion channels modulated by ethanol are $GABA_A$, NMDA and nAChRs.

Ethanol and GABAergic System

Modulation of the GABAergic system is a major mechanism uderlying several behavioral actions of acute and chronic ethanol exposure. An increase in ethanol-induced GABAergic neurotransmission is due to elevation of endogenous GABAergic neuroactive steroids, presynaptic release of GABA, and dephosphorylation of $GABA_A$ receptors leading to high GABA sensitivity. Several behavioral actions of ethanol involve direct or indirect potentiation of $GABA_A$ receptor functions. For example, the selective $GABA_A$ antagonist, RY023, interferes with ethanol's rewarding, sedative and motor impairing actions and α_5 and δ subunit containing $GABA_A$ receptor knockout mice exhibit reduced ethanol consumption (Cook et al., 2005). We have also shown that intra-striatal microinfusion of the partial inverse agonist of the central benzodiazepine receptor, Ro 15-4513, significantly attenuated ethanol ataxia by binding with high affinity to δ-subunit of $GABA_A$ receptors (Meng and Dar, 1996).

Acute ethanol administration has been shown to increase the synthesis, expression and translocation of PKC_β, PKCγ and PKCε isoforms (Kumar et al., 2006). Indirect activation of $GABA_A$ receptors by ethanol occurs through PKC and PKA mediated phosphorylation of the receptor protein (McDonald and Moss, 1997; Brandon et al., 2000).

As has been demonstrated in several studies the genetic deletion or knockout of some isoforms of PKC disrupt ethanol's effects. For example, PKC_{δ} knockout mice are insensitive to ethanol's ataxia and PKCγ knockout mice showed less anxiolytic effects of ethanol (Bowers et al., 2001; Choi et al., 2008). Chronically, ethanol elicits $GABA_A$ receptor internalization, reduction in sensitivity that underlie ethanol related tolerance, dependence and withdrawal hyperexcitability (Roca et al., 1990; Tehrani and Barnes, 1997). Besides its main action on the $GABA_A$ receptor, ethanol also augments baclofen-induced IPSPs at $GABA_B$ receptor subtypes (Federici et al., 2009).

Ethanol and Glutamate Receptors

Generally, ethanol inhibits all ionotropic glutamate receptors, though the effect and degree of inhibition varies with the kinetic state of the receptors in question. In the case of AMPA receptors, ethanol induces an increased desensitization rate (Moykkynen et al., 2003) and, for NMDA, a decreased burst frequency and intra-burst open channel time by ethanol (Wright et al., 1996) that leads to channel inhibition. Acute as well as chronic ethanol alters the function of NMDA receptors by inhibiting Ca^{2+} influx through the NMDA receptor-coupled ion channel. Chronic ethanol exposure, in contrast, results in an upregulation of the NMDA receptor/ionophore complex (Iorio et al., 1992; Snell et al., 1993). Acute ethanol also increases extraneuronal adenosine levels which in turn inhibits glutamate release (Clark and Dar, 1989). Overall, ethanol acts as a functional antagonist at the glutamate receptor and could block the excitatory transmission of glutamate at the postysynaptic receptor level.

Ethanol's Modulation of Adenosine System

The adenosinergic modulation of some of central effects of ethanol have been documented in several studies (Dar, 1990; Nagy et al., 1991; Meng and Dar, 1996). Previous work in this laboratory demonstrated that the brain adenosine system modulates ethanol ataxia, primarily via A1 receptors in three key motor areas i.e. cerebellum, striatum and motor cortex (Dar, 1990; Barwick and Dar, 1998). Evidence came from the observation that direct intra-striatal and cerebellar microinfusion of the A1-selective agonist, N6-cyclohexyladenosine (CHA), dose-dependently accentuated ethanol ataxia. Furthermore, an A1-selective antagonist but not the A2-selective antagonist, DPCPX not only antagonized ethanol ataxia but also reduced the accentuation produced by CHA, suggesting a role for tonic adenosinergic modulation of ethanol ataxia. The study revealed the involvement of the pertussius toxin-sensitive Gi/Go protein and cAMP as key signaling agents in ethanol ataxia since intra-striatal pretreatment with pertussius-toxin markedly antagonized the accentuation of ethanol ataxia by CHA probably by catalyzing the ribosylation of the α-subunit of Gi/Go. As a result there was activation of adenylyl cyclase and an increase in cAMP that overcame ethanol ataxia. This was further confirmed in studies where intra-striatal or cerebellar microinfusion of cAMP or its analog, cpt-cAMP, markedly antagonized ethanol ataxia (Dar, 1997). Conversely, ethanol directly increased the ADP ribosylation of Gi/Go protein indicating that an increase in Gi/Go protein activity results in inhibition of adenylyl cyclase and dimunition of cAMP levels leading to ataxia (Dar and Meng, 2004).

Ethanol and Voltage gated ion channels

Voltage gated Na^+/K^+ channels respond to ethanol at concentrations less than 100mM. As for K^+ channels, the G-protein coupled inward rectifying K^+ channel (GIRK), the large conductance Ca-activated K^+ channels (BK) and shaw 2 voltage gated channels (Kv3) (Dopico et al., 1998; Kobayashi et al., 1999; Lewohl et al., 1999) are primarily modulated by ethanol. Ethanol-induced modulation of the BK channels are activated at intracellular Ca^{2+} less than 10μM and are inhibited at intracellular Ca^{2+} more than 10μM. Ethanol depresses Na^+-induced action potential generation by binding preferentially to nAChRs channel in the inactivated state rather than in the open state (Xiao et al., 2008). Besides, voltage gated Ca^{2+} channels can also serve as a target site for ethanol action. Pretreatment with non-selective Ca^{2+} channel inhibitor, NP 078585, can attenuate rewarding/reinforcing properties of ethanol (Newton et al., 2006).

Ethanol and Enzyme Targets

Ethanol modulates numerous kinases, which in turn regulate and alter other cellular proteins or behavioral responses. By inhibiting adenosine uptake, ethanol augments extracellular adenosine levels, which can excite A_{2A} receptors leading to cAMP production and PKA activation. Once activated, PKA can trigger various cellular changes such as modulation of ion channel activity (Melis et al., 2002). Ethanol-induced phosphorylation of PKA/PKC and tyrosine kinases (i.e. Src and Fyn) are key regulators for the activity of ligand-gated ion channels in particular, $GABA_A$ and NMDA-coupled channels (Wafford et al., 1991; Anders et al., 1999; Alvestad et al., 2003). Additionally, ERK, a member of the mitogen-activated protein kinase family also constitutes an important target for ethanol reinforcing action as inhibition of this kinase can greatly augment ethanol self-

administration (Faccidomo et a.l, 2009). With one single injection, ethanol can trigger CREB phosphorylation, which is implicated in long-lasting synaptic plasticity (Camarini and Hodge, 2004). Furthermore, ethanol-induced release of GABA at Golgi cell-Purkinje cell synapse is regulated by adenylyl cyclase-PKA signaling since AC and PKA antagonists can blunt GABA release (Kelm et al., 2008).

Ethanol and Nitric Oxide

Endogenous NO is an inorganic, highly diffusible, lipid soluble gas with a biological half-life of only a few seconds. Nitric oxide is synthesized in mammalian systems by the action of one of three NOS isoforms: inducible (iNOS), endothelial (eNOS) and neuronal (nNOS) which catalyze the conversion of l-arginine to NO and l-citrulline using several and cofactors (i.e. FMN, FAD, NADPH, BH4) (Stuehr and Griffith, 1992). Nitric oxide is a highly reactive and unstable chemical and is immediately degraded to nitrite (NO_2^-) and nitrate (NO_3^-) (Knowle & Moncada, 1994). Nitric oxide can also react with superoxide (O_2^-) to form a highly toxic oxidizing reagent, peroxynitrite (Beckman et al., 1990). Thus, excessive NO production, albeit contributed largely by iNOS imparts a cytotoxic action (Calabrese et al. 2007). Virtually all neurons express the housekeeping isoform nNOS and possess the mRNA for iNOS which is translated under pathological conditions (Snyder, 1994). In the CNS, NO serves as a biological mediator of neurotransmitter release, gene expression, cell survival, blood flow, cognition, and immunomodulation.

The effect of ethanol on NOS enzymes and subsequent NO release varies depending upon the site, dose and duration of ethanol exposure. Consequently, the existing literature contains data showing increases (Chandler et al., 1997), decreases (Zima et al., 1998) and no alteration (Ikeda et al., 1999) in NOS activity following acute or

chronic ethanol treatment. Despite the variability of the action of ethanol on the NO system, NO appears to mediate several effects of ethanol. Nitric oxide plays a key role in ethanol preference as L-NO arginine, a potent inhibitor of NOS, reduces ethanol self-administration without altering normal feeding behavior (Calapai et al., 1996). Another study shows that pretreatment with 7-nitroindazole, a selective nNOS inhibitor, blocked ethanol-induced locomotor sensitization responses and condition placed preference in mice (Itzhark and Martin, 2002).

NO has also been implicated in the production of tolerance to ethanol-induced motor incoordination as inhibitors of cGMP, such as LY83583 and ODQ, prevented the development of tolerance to ethanol's action while the cGMP analogue, 8-bromo-cGMP, and the NO donors, SNAP or sodium nitroprusside, increased ethanol tolerance (Wazlawik and Morato, 2003). Similarly, nNOS knockout rats were less sensitive to ethanol's sedative (Spanagel et al., 2002), hyperlocomotion action (Itzhak and Anderson, 2008) and did not develop rapid tolerance to ethanol- induced hypothermia versus wild type animals (Spanagel et al., 2002).

Nitric oxide is also critical in the ethanol withdrawal response. Inhibition of NOS ameliorates some symptoms of ethanol withdrawal as shown in a study where pretreatment with the nNOS inhibitors, N-nitro-L-arginine methyl ester (L-NAME) and 7-nitroindazole (7-NI), significantly controlled ethanol withdrawal signs (hyperlocomotion, audiogenic seizures) while l-arginine administration potentiated ethanol's withdrawal responses ((Uzbay & Erden, 2003). Nitric oxide protects the neuronal population in the hippocampus, the dentate gyrus and the neocortex against ethanol-induced toxicity as nNOS knockout (-/-) mice suffer more alcohol-induced toxicity than wild type mice

(Bonthius et al., 2006). It has been shown that chronic ethanol exposure to maternal rats reduced NOS expression in the hippocampus of young offspring thus providing a clue to the underlying ethanol-related developmental retardation and abnormalities (Jang et al., 2005). Besides its action on nNOS, ethanol pretreatment also inhibits cytokine-induced iNOS activity in immortalized astrocytes (Wang and Sun, 2001). Ethanol can also markedly inhibit highly expressed NOS in cerebellar granule and basket cells (Fataccioli et al., 1997).

Nicotine-Ethanol Interaction

Nicotine and alcohol are the two most damaging and addictive substances in our society that are very frequently used by the same individuals. It is generally true that a significant proportion of alcoholics smoke and are also nicotine-dependent (Littleton et al., 2007). It is equally true that for people who are dependent upon alcohol, tobacco is one of the most commonly co-abused substances (Ceballos 2006). The concurrent use of nicotine and alcohol in humans has long been documented by several epidemiological studies (Hays et al., 1999; Anthony et al. 2000; Meyerhoff et al., 2006). Nearly 80-90% of alcoholics smoke regularly (Pomerleau et al., 1997; Romberger and Grant, 2004) and alcoholism is approximately 10 times more prevalent in smokers than nonsmokers (DiFranza and Guerrera, 1990).

Generally, consensus exists among researchers that the primary cause of this co-addiction is the fact that nicotine and ethanol synergistically release more dopamine in the reward pathway than either drug alone (Soderpalm et al., 2000; Narahashi et al., 2001). It has also been suggested that there are other sites wherein these drugs modulate each other's physiological effects that may also contribute to co-addiction. However, the precise

mechanism(s) of co-abuse is/are not known probably because of the complex functional relationship between the two drugs of abuse. Within the CNS both drugs modulate the action of each other in a bi-directional manner. For example when ethanol acts as a co-agonist it augments nicotine's locomotor stimulatory, rearing (Lapin et al., 1995; Watson and Little, 1999) and rewarding activity (Korkosz et al., 2006a). Conversely, ethanol also exhibits antagonistic functional interactions with nicotine. For example, ethanol reduces nicotine-induced seizures (Korkosz et al., 2006b) and improvement in cognitive functions (Rezvani and Levin, 2002). Chronically, ethanol abolishes the hypothermic effects of nicotine (Majchrzak and Dilsaver, 1992). *In vitro,* ethanol prevents nicotine-induced inhibition of Purkinje neurons as measured by extracellular single-unit recording (Yang et al., 1999). Similarly, nicotine also facilitates or counters ethanol related actions. For example, subchronic nicotine treatment leads to increased ethanol consumption in rats (Larsson and Engel, 2004) and exposure of young rats to nicotine during adolescence results in higher ethanol consumption at adulthood (Tsui et al., 2001). Nicotine attenuates the ethanol-induced increase in glucose utilization in mouse cerebellar tissue (Anwer and Dar, 1995). In cerebellar cortical and granule cells, nicotine decreases ethanol-induced caspase 3 activity and subsequent cytotoxicity (Tizabi et al., 2005). Nicotine blocks ethanol-induced impairment in contextual and cued fear conditioning (Gulick and Gould, 2008).

Ethanol and Nicotinic Acetylcholine Receptors

Several behavioral actions of ethanol are mediated by nAChRs. For example, ethanol-induced dopamine release in the reward pathway and locomotor stimulation require activation of nAChR as the non-selective nAChR antagonist, mecamylamine,

reduced both actions of ethanol (Soderpalm et al., 2000). Ethanol, acting as co-agonist at nAChRs, facilitates the binding of nicotine (Tonner et al., 1992), and stabilizes the open state of the nAChR channel (Wu et al., 1994). Chronic ethanol administration increases nicotine binding sites in the brain (Collins et al., 1996) but decreases binding sites in M10 and SH-SY5Y neuroblastoma cells (Gorbounova et al., 1998). The action of ethanol's action on nAChR subtypes is differential as evidenced by the observation that ethanol potentiates the agonist-induced current at $\alpha_4\beta_2$ subtype receptors and inhibits the α_7-induced current in recombinant preparation of *Xenopus Lae vis oocytes* (Narahashi et al., 1999; Zuo et al., 2002). Since $\alpha_4\beta_2$ and α_7 are the predominant nAChR sutype, a detailed account of the role of each subtype in ethanol's action is given below.

Role of α4β2 Subtype in Ethanol's Action

The role of $\alpha_4\beta_2$-containing nAChRs in contributing to ethanol action is controversial. Some *in vitro* studies also support the idea that ethanol potentiates the agonist-induced current at $\alpha_4\beta_2$, $\alpha_3\beta_2$, $\alpha_2\beta_2$ nAChRs in rat cortical neurons (Aistrup et al., 1999) and *Xenopus oocytes* (Covernton & Connolly, 1997). Tritto et al. (2004) have shown the participation of α_4 subunit containing nAChRs in mediating ethanol's increase in locomotor activity. Previously, we have demonstrated that intracerebellar microinfusion of nicotine attenuates ethanol ataxia in a dose-dependent fashion through a glutamate-nitric oxide-cGMP signaling pathway (Dar et al., 1993; Dar et al., 1994; Al-Rejaie and Dar, 2006b, c). Conversely, data exist that do not support any role of $\alpha_4\beta_2$-containing nAChR subtypes in mediating the release of dopamine by ethanol, locomotor stimulation and rewarding effect also was reported (Le et al., 2000; Larsson et al., 2002; 2004). According to Larsson et al. (2002) selective antagonism using $\alpha_4\beta_2$ receptor antagonist, DHβE, as well as by selective

α_7 blocker, methyllycaconitine, does not diminish ethanol-induced locomotor stimulation and dopamine release in nucleus accumbens. Rather a negative allosteric modulator of nAChRs, mecamylamine, attenuates these actions of ethanol by a mechanism different from direct inhibition of $\alpha_4\beta_2$ or α_7 subtypes function (Larsson et al., 2002).

Role of α_7 Subtype in Ethanol's Action

Ethanol-induced dopamine release and hyperlocomotor activities are mediated through non-$\alpha_4\beta_2$ and non-α_7 containing nAChRs as selective antagonists at these subtypes failed to prevent these actions of ethanol (Le et al., 2000; Larsson et al., 2002; 2004). On the other hand, varenicline, a novel α_7-selective full agonist and $\alpha_4\beta_2$ partial agonist, has proven effective in decreasing ethanol consumption in laboratory animals (Steensland et al., 2007). Varenicline (Chantax®) has recently been approved and is clinically available for smoking cessation. Varenicline also improved ethanol-induced impairment in contextual (Gulick and Gould, 2008) and cue associative learning (Steensland et al., 2007) indicating that the nAChR α_7 subtype might have some contribution in some behavioral actions of ethanol. Moreover, α_7 knockout mice exhibited heightened sensitivity to ethanol-induced loss of rightening reflex (LORR) and an increase in open-field activity (Bowers et al., 2005). These findings were supported by the observation that genotypic alcohol sensitive long-sleep mice have significantly reduced density of cerebellar α_7 subtype (de Fiebre et al., 1987). Consequently, these mice display greater sensitivity to the depressant effects of ethanol such as sedation and hypnosis (Bowers et al., 2005). Since LORR is a consequence of ethanol-mediated depression of cerebellar Purkinje cells, it has been proposed that cerebellar α_7 subtypes tonically oppose ethanol-induced LORR (Bowers et al. 2005). Despite the given role of $\alpha_4\beta_2$ and α_7 subtype

in nicotine-ethanol, no study to the best of our knowledge has looked into the participation of these subtypes in the mediation of ethanol ataxia that results from ethanol-induced disruption in rhythmic firing of cerebellar neurons (Carta et al., 2004; Botta et al., 2007).

CHAPTER TWO: EXPERIMENTAL PROCEDURES

Animals

Male mice (Charles River, Raleigh, NC) of CD-1 (ICR) strain, weighing 22-24 g and 5-6 weeks old were used in all studies reported. This inbred strain of mice at this age and body weight are considered adult (based on consultation with Animal Care Veterinarian) and have been used in the ongoing research since 1980. These mice exhibit a very consistent ataxic response due to ethanol. Older and heavier animals do not provide consistent responses in their Rotorod behavior. Upon arrival, the animals were housed 8 per cage, and fed commercial pellet food and water ad libitum. The animals were kept in housing quarters under controlled humidity (60-80%) and temperature 22-24^{o}C and maintained on a 12-h light/dark cycle, with lights on at 07:00 h. The animals were allowed to acclimatize to their housing conditions at least for 24 h before survival stereotaxic surgery. Following implantation of the permanent guide cannulas for drug microinfusion, each animal was housed individually in a plastic cage. The animals were used only once in the Rotorod experiment. The use of animals in the present study was in accordance to the laboratory animal use protocol approved by the University's Animal Care and Use Committee consistent with the NIH *Guide for the Care and Use of Laboratory Animals.*

Drugs Used in the Project

The following drugs and concentration were used in the study: (-)-nicotine-di-L-tartrate (5ng); $\alpha_4\beta_2$-selective nAChR subtype agonist, N-methyl-4-(3-pyridinyl)-3-buten-1-amine (RJR-2403; 31 62, 125, 500 ng); $\alpha_4\beta_2$-selective nAChR subtype antagonist, dihydro-β-erythroidine hydrobromide (DHβE; 125, 250, 500, 750 ng), α_7 selective agonist, N-(3R)-

1-Azabicyclo[2.2.2]oct-3-yl-4-chlorobenzamide (PNU-282987 2.5µg, 250 ng, 25 ng); α_7 selective antagonist, methyllycaconitine (MLA, 3, 6, 12ng). RJR-2403 and PNU-282987 were purchased from Tocris (Ellisville, MO, USA) whereas Sigma-Aldrich (St. Louis, MO, USA) was the source of nicotine, DHβE and methyllycaconitine. Stock solutions of nicotine, RJR-2403, DHβE and methyllycaconitine were prepared in artificial cerebrospinal fluid (aCSF) containing in (mM): NaCl (127.65); KCl (2.55); $CaCl_2$ (0.95); $MgCl_2$ (0.94); $Na_2S_2O_5$ (0.05) at pH 7.4 and PNU-282987 was dissolved in 100% dimethylsulfoxide (DMSO). To maintain the stability of drug solutions, aliquots of the stock drug solutions were prepared and stored at -70°C while the working concentrations of drugs were either prepared on the day of the experiment or a day before and kept at -70°C. Ethanol solution (10% v/v) was prepared from absolute ethanol, diluted with 0.9% sterile saline and was administered i.p. at a dose of 2g/kg (0.2ml/10g). Chloral hydrate was diluted in sterile water to achieve the desired dose of 450mg/kg (0.1ml/10g of body weight; i.p.). The ethanol dose of 2g/kg results in a blood alcohol concentration of about 185 mg/dL (~ 38 nM) based on previous studies from this laboratory (Dar, 1988). This dose consistently produces motor impairment with no loss of righting reflex and without overt sedation or hypnosis. It is comparable to the blood alcohol concentrations (22 to 33 nM = 100 – 150 mg/dL) that occur following moderate social drinking in humans who experience consistent psychomotor impairment but no sleep or overt sedation.

Synthesis of Oligonucleotides

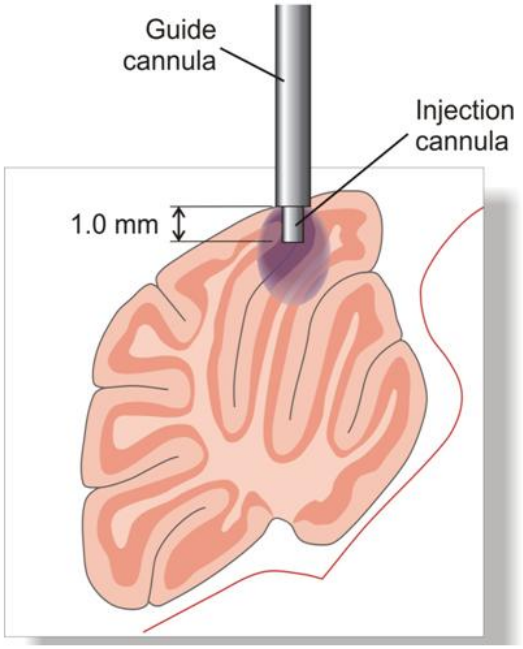
Oligonucleotides were custom-synthesized, containing a phosphorothioate backbone and purified by Integrated DNA Technologies, Inc. (IDT, San Diego, CA, USA). Sequences of $\alpha_4\beta_2$ and α_7 subtypes antisense and missense, obtained from the published

literature (Yu and Role, 1998; Bitner et al., 2000), were as follows: $\alpha_4\beta_2$ antisense 5`-(CGAATTGGCCATGGTGAAGC); missense 5`-(CAGATTGGCCATGGTGAGAC); α_7 antisense 5`-(GCATCAGCGCCCGGA)-3`; missense 5`-(GCAACAGAGCCAGGA)-3`. The oligos were dissolved in an appropriate volume of sterile water to provide a final concentration of 200nM.

Stereotaxic Surgery for Cannula Implantation

Mice, under chloral hydrate (450 mg/kg, i.p.) anesthesia and Ketorolac analgesia (2mg/kg; s.c), were stereotaxically implanted with a permanent indwelling stainless-steel guide cannula for microinfusion of drugs directly into the cerebellum (Fig. 4). Anesthetized animals were placed in a stereotaxic apparatus (Model 900; David Kopf Instruments, Tujunga, CA, USA) and the guide cannula implanted according to coordinates of Slotnik and Leonard (1975). The coordinates for the cerebellar cannulation were: AP,-6.4 mm (bregma); ML, ± 0.8 mm, DV,-1.0 mm: from the skull surface. The guide cannula was constructed from 22-gauge stainless steel tubing, cut to a length of 10 mm and lowered to the desired depth through the appropriately located craniotomy holes. They were anchored with fast drying carboxylase-cement, Durelon® (Premier Dental Products Co., Norristown, PA, USA) to the cranial surface that had been scraped clean of periosteum. A minimum period of at least 4 days was allowed for the recovery of mice from the surgical trauma and the anesthesia before their use in behavioral experiments. The surgical implantation of the indwelling cannula was carried out under aseptic conditions. The cranial surface was routinely cleaned by swab sticks of povidone-iodine solution, (The Clinipad Co., Rocky Hill, CT, USA). The guide cannula, drill burs and surgical tools were autoclaved prior to use. Currently, 95% of animals successfully recover post-operatively for use in behavioral

FIGURE 4: Diagram of sagittal view of stainless steel guide annula surgically implanted into anterior lobe region of mouse cerebellum. The injection cannul protrudes one mm beyond the ventral tip of the guide cannula


Guide
cannula
Injection
cannula
1.0 mm

experiments. To minimize possible infection, aseptic surgical techniques were followed consistent with the approved animal use protocol. All animals received subcutaneous (s.c.) 3000 units of Durapen®, (VEDCO Inc, MO, USA), a combination of benzathine and procaine penicillin G suspension immediately after surgery. Animals also received injections (2mg/kg, s.c.) of an analgesic, ketorolac tromethamine (Abbot Laboratories, North Chicago, IL, USA) before and four hours after surgery.

Rotorod Treadmill - motor coordination

The degree of motor impairment was determined using a standard mouse Rotorod treadmill (UGO Basile, Verese Italy), calibrated for a fixed speed of 24 rpm (Dar, 1990; Smith and Dar, 2007). Mice were allowed to acclimatize to the treadmill by placing them on it 2-3 times immediately prior to the actual experiment. It was important to test each mouse for its motor coordination before its use in Rotorod study in case of an inborn cerebellar defect. The screening test now used in our laboratory requires each animal to remain on the Rotorod for 180 s. Based on repeated observations for over 25 years, less than 3% of all mice tested fail to meet this criterion. Only successfully screened animals received pretreatment (aCSF/oligonucleotides/or nicotinic drugs) followed 5 min (in case of drugs) and 16 h (in case of oligonucleotides) by the i.p. injection of the test dose (2g/kg) of ethanol. The index of motor coordination was always evaluated every 15 min for 60 min starting immediately after ethanol administration. To avoid the influence of diurnal variation on the motor behavior of animals, all Rotorod experiments were conducted between 8:00 am and noon.

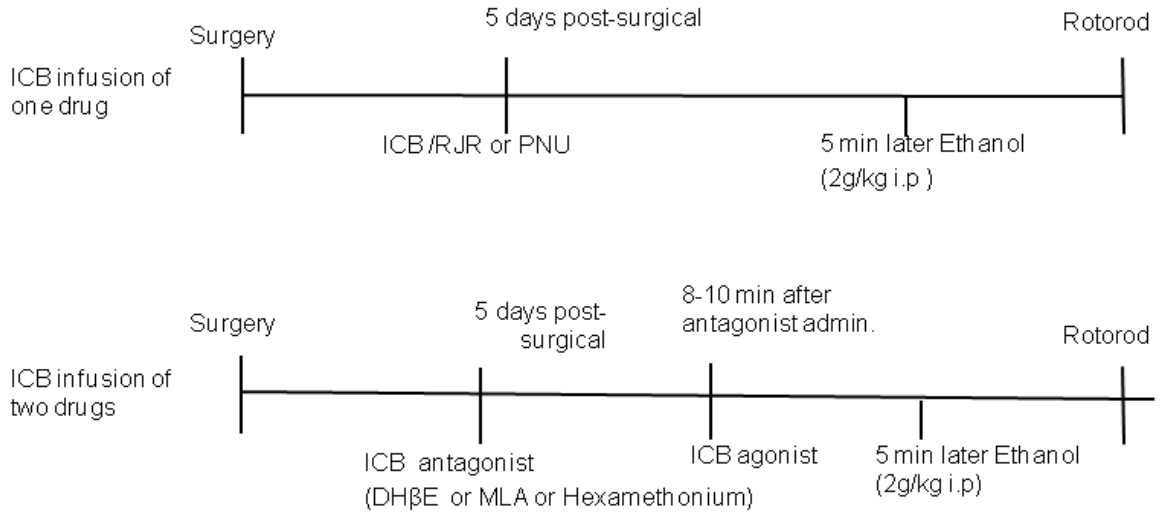
Intracerebellar Microinfusion of Drugs

In this dissertation research, all nicotinic drugs including, $\alpha_4\beta_2$ and α_7 antisense and missense oligonucleotides were microinfused directly into the culmen of the anterior lobe of the cerebellum through an injector cannula (11mm length, 30-gauge) connected via a polyethylene tube (Clay Adams, Parsippany, NJ, USA) to a 25 µl Hamilton microsyringe. The microsyringe was fitted onto a microinfusion pump (Model 22, Harvard Apparatus, South Natick, MA, USA), which regulated the constant infusion rate. The injector cannula was inserted into the guide cannula and was allowed to protrude 1 mm beyond the lower tip of the guide cannula. An air bubble introduced in the PE-10 tube helped track the flow of drug solution. Unless otherwise state, the animals received an ICB microinfusion of one drug followed 8-10 min later by ethanol administration (Fig.5). The Rotorod test was performed 15 min after the ethanol injection. In the case of two ICB drug treatments, first, the animals received an ICB microinfusion of the agonist, followed by the antagonist after 5 min interval and then the Rotorod test was conducted (Fig. 5). Oligonucleotides were also administered under the same setting with the following protocol: 200nM, twice a day for 4 days. Ethanol as stated above was administered i.p. throughout this project.

Protocol for Chronic Study

Presented in Figure 6 is a brief overview of the experimental protocol used to study the consequences of repeated intracerebellar microinfusion of nicotinic agonists and antagonists as well as the development of cross-tolerance between repeated RJR-2403 or PNU-282987 and acute ethanol induced ataxia. The details of the experiments are presented below:

FIGURE 5: Schematic diagram showing the drug administration protocol in acute studies

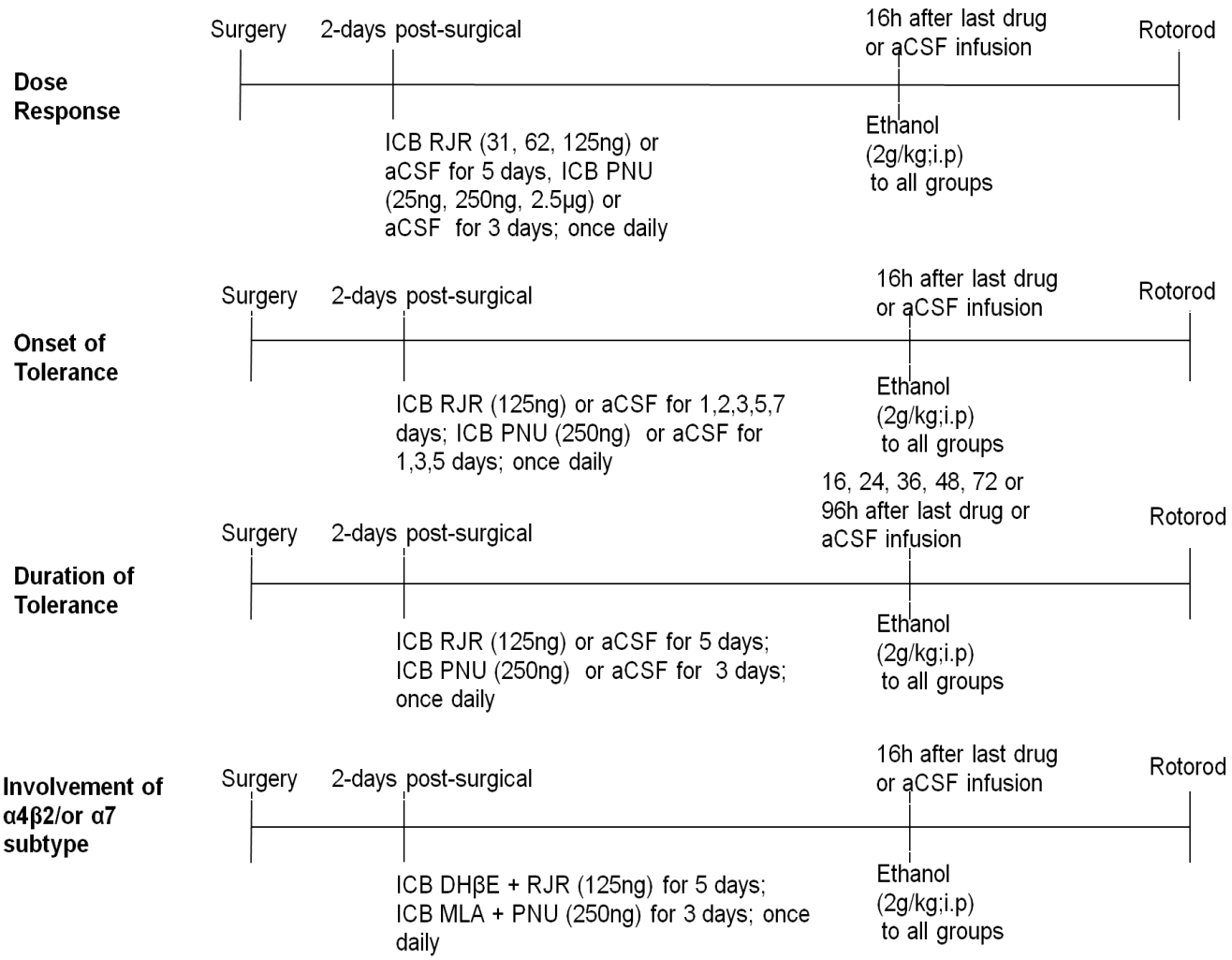

ICB infusion of one drug
Surgery
5 days post-surgical
Rotorod
ICB /RJR or PNU
5 min later Ethanol (2g/kg i.p)
ICB infusion of two drugs
Surgery
5 days post-surgical
8-10 min after antagonist admin.
Rotorod
ICB antagonist (DHβE or MLA or Hexamethonium)
ICB agonist
5 min later Ethanol (2g/kg i.p)

Dose-Response Relationships. In order to determine if the development of tolerance to acute ethanol-induced ataxia following repeated RJR-2403 (31, 62,125ng) and PNU-282987 (25ng, 250ng, 2.5µg) was dose dependent, α4β2 and α7 selective agonists were microinfused directly into the cerebellar cortex once daily for 7 days (RJR-2403) and for 5 days (PNU-282987). Sixteen hours after the last RJR-2403 and PNU-282987 intracerebellar microinfusion, the test dose of ethanol was administered as explained in Rotorod procedure and motor incoordination was evaluated 15-60 min post-ethanol injection.

Onset of Tolerance. To determine the time when the development of cross-tolerance is first observed following repeated intracerebellar microinfusion, an optimum dose of RJR-2403 and PNU-282987 was selected from dose-response data. The RJR-2403 dose of 125ng was selected based on the dose-response experiments. This dose was routinely used in determining the “onset and the duration of the cross-tolerance” between RJR-2403 and acute ethanol ataxia. Similarly, the dose of PNU-282987 selected was 250ng that was used in experiments to determine the “onset and duration of the cross-tolerance”. Animals were repeatedly treated with ICB RJR-2403 (125ng for 1, 2, 3, 5, 7 days) and PNU-282987 (250ng for 1, 3, 5 days). Finally, 16 h after the last agonist injection, the test dose of ethanol was administered and ethanol ataxia was evaluated.

Duration of Tolerance*.* After determination of the time for onset of development of cross tolerance, we sought to find out the total time for which cross-tolerance persisted. Again, animals were microinfused with RJR-2403 (125ng; once a day for 7 days) and PNU-282987 (250ng; once a day for 5 days). Ethanol ataxia was evaluated starting 16 h after the last drug treatment and continued until the effect of the drug tapered off.

FIGURE 6: Schematic diagram showing the protocol for chronic study


Dose Response
Surgery
2-days post-surgical
16h after last drug or aCSF infusion
Rotorod
ICB RJR (31, 62, 125ng) or aCSF for 5 days, ICB PNU (25ng, 250ng, 2.5µg) or aCSF for 3 days; once daily
Ethanol (2g/kg;i.p) to all groups
Onset of Tolerance
Surgery
2-days post-surgical
16h after last drug or aCSF infusion
Rotorod
ICB RJR (125ng) or aCSF for 1,2,3,5,7 days; ICB PNU (250ng) or aCSF for 1,3,5 days; once daily
Ethanol (2g/kg;i.p) to all groups
Duration of Tolerance
Surgery
2-days post-surgical
16, 24, 36, 48, 72 or 96h after last drug or aCSF infusion
Rotorod
ICB RJR (125ng) or aCSF for 5 days; ICB PNU (250ng) or aCSF for 3 days; once daily
Ethanol (2g/kg;i.p) to all groups
Involvement of α4β2/or α7 subtype
Surgery
2-days post-surgical
16h after last drug or aCSF infusion
Rotorod
ICB DHβE + RJR (125ng) for 5 days; ICB MLA + PNU (250ng) for 3 days; once daily
Ethanol (2g/kg;i.p) to all groups

Participation of nAChR Subtypes in Tolerance Development. To confirm the participation of specific nAChR subtypes in the development of cross-tolerance between nAChR subtypes $\alpha_4\beta_2$ and α_7 and the acute ethanol ataxia, mice were pretreated with $\alpha_4\beta_2$- and α_7-selective antagonists, DHβE and methyllycaconitine, respectively, followed by the agonist drugs RJR-2403 or PNU-282987 as before. Sixteen hours after the last microinfusion of RJR-2403 or PNU-282987, motor incoordination was assessed as before.

Histology

It was important to confirm the accuracy of guide cannula implantation as well as of the drug microinfusions. Therefore, at the conclusion of each behavioral experiment, 100 nL of Fast Green dye was microinfused into each animal through the guide cannula. Mice were euthanized by cervical dislocation and decapitation under light Isoflurane anesthesia (IsoFlo; Abbott Laboratories, North Chicago, IL, USA). The guide cannulas and the brains were carefully removed and the correctness of microinfusion site was verified by visual observation of the presence of dye in the cerebellar anterior lobe region. Only animals in which the cannula placement was histologically verified as correct were included in the data analysis. The brains were frozen at -20°C and subsequently, 20µm thick coronal sections were obtained with a Tissue-Tek II cryostat (Mills Laboratories, Naperville, IL, USA). The sections were mounted onto a clean glass slide, allowed to air dry and then stained with cresyl violet. The sections were examined under light microscope to assess the integrity of cerebellar cortex after repeated nicotinic drugs or oligonucleotide treatment and to confirm the accuracy of drug microinfusion site. Histological photomicrographs were taken with a spot Insight Digital Camera connected to an Olympus Bx51 microscope at 12.5x. In the present dissertation research 100% of the

cannula implantation was successful. Based on the examination of histologic photomicrographs of cerebellar tissues from randomly selected mice, a minimal variation between and within groups and treatments in the drug dispersion sites of microinfusions and the extent of tissue damage due to cannula implantation was observed. The histological data also confirmed the drug dispersion following its intracerebellar microinfusion remained confined to the cerebellar tissues consistent with previous studies (Meng and Dar, 1996).

Immunoblot Experiment

Preparation of Membrane Fraction. For preparation of membrane proteins, oligonucleotide-treated mice were sacrificed by cervical dislocation and decapitation. After careful removal and transfer of brains to an ice cold glass plate surface, cerebellar tissues were cut out around the site of the guide cannula implantation. The cerebellar tissues from 5 animals were pooled to increase the amount of the available proteins in the tissue sample. Six volumes of ice cold Tris buffer (20 mM Tris, 2 mM EDTA, 250 mM sucrose) were added and the samples were homogenized using a glass Teflon homogenizer. Homogenates were centrifuged at 2000 x g for 5 min at 4°C, the resultant supernatants were re-centrifuged at 22,000 x g for 30 min. Finally, the pellets were resuspended in Tris buffer, the suspension was well shaken, divided into aliquots and stored at -20°C until the day of the experiment. Protein measurements were made using the BCA protein assay (Thermo Fisher Inc. Rockford, IL, USA).

Western Blot Analysis. Eighty µg of membrane protein were size fractionated using 10% SDS-PAGE, followed by transfer onto nitrocellulose at 100V for 100 mins. The membranes were blocked with 1x casein (Vector Laboratories, Burlingame, CA, USA) in

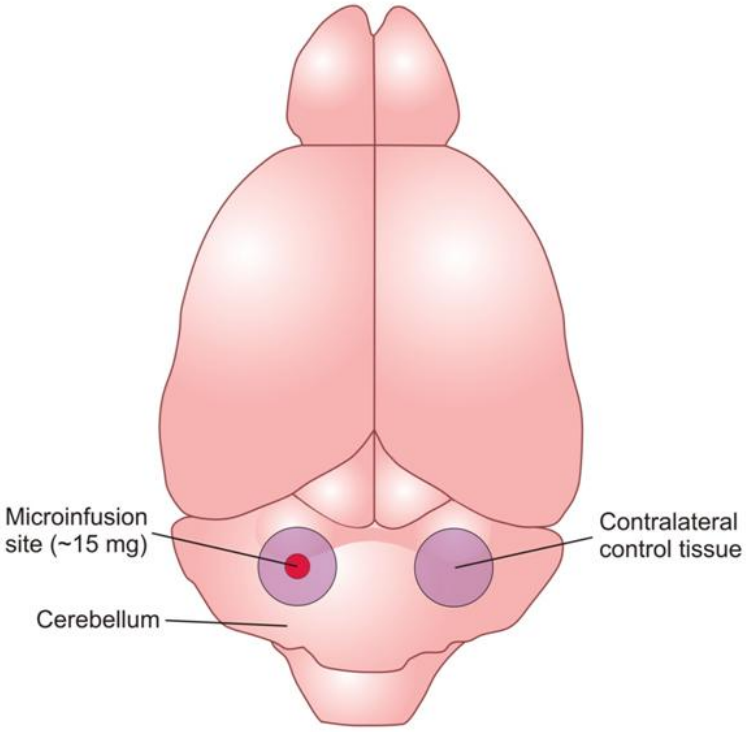
Tris-buffered saline-T [20 mM Tris, 500 mM NaCl, 0.1% Tween 20 (TBS-T), pH 7.6] for 4 hours at room temperature followed by incubation with primary rat antibody directed against the α_4 and the α_7 subunit of nAChRs (Covance Research Products, Berkley, CA, USA) at 1:500 dilution with TBST-casein at 4°C overnight. After washing with TBST-T for 3x5min, membranes were probed with secondary goat anti-rat horseradish peroxidase conjugated antibody in a 1:5000 dilution (Santa Cruz, Biotechnologies, Inc. Delware, CA, USA) for 2 hours at room temperature. This was followed by 3x5 min wash with TBST-T. Specific protein bands were visualized using the ECL western blotting detection reagent and the Versadoc Imaging System (Bio-Rad Laboratories, Richmond, CA, USA). The peak density of the bands was measured.

Biochemical Study

Nitric oxide Assay. Intracerebellar concentrations of highly unstable nitric oxide (NO) were measured indirectly by reading its reactive metabolites (i.e. nitrite and nitrate [NOx]) using DAN fluorimetric methods (Rao et al., 1998; Al-Rejaie & Dar, 2006a,b).

Cerebellar Tissue Preparation. All glass and plastic wares used in the procedure were washed thoroughly with de-ionized water and were air dried before the experiment to minimize interference from the environmental NO. Following the ICB nicotinic drug treatment and ethanol or saline i.p. administration, mice were euthanized 15 min post-ethanol injection, by cervical dislocation and decapitation. This time point was selected based on the ethanol response curve in the Rotorod results wherein ethanol ataxia peaked at 15 min post-injection. Brains were removed from the skull and placed on an ice-cold glass plate.The cerebellar tissue surrounding the guide cannula mark was dissected out (Fig. 7) and immediately frozen in liquid nitrogen. The frozen tissues were

FIGURE 7: Schematic diagram showing the area where both treated and contralateral cerebellar tissue was dissected


Microinfusion
site (~15 mg)
Contralateral
control tissue
Cerebellum

weighed in a tared tube and homogenized in eight volumes of Tris EDTA buffer pH 7.4 using a Polytron tissue homogenizer (Polytron Model PT 10/35, Brinkman Instruments, Westbury, NY, USA). Typically, samples obtained from each animal weighed 15-30mg. All samples were centrifuged at 4000xg for 20 min. The crude filtrate was ultracentrifuged through 10,000 MW cutoff microcentrifuge filters (Microcon® YM-10, Amicon® Bioseparations, Millipore Co., Bedford, MA, USA) at 16,000xg for 60 min at 4°C. The clear ultrafiltrate obtained was free of hemoglobin and other protein contamination that have been reported to interfere with the assay results (Fernandez-cancio et al., 2001). Detection of cerebellar NOx concentration was done in a 2-step procedure. First, the tissue nitrate was enzymatically converted to nitrite by NADPH-dependent nitrate reductase. Second, the nitrite was allowed to react with DAN to form a fluorescent product, 2, 3-naphthotrazole, that was measured fluorometrically. The actual assay procedure involved pipetting 100 µl of the sample cocktail containing, 20 µl of 750 µM G-6-P, 20 µl of G-6-PDH (48 mU), 20 µl of 3 µM NADPH, 20 µl of nitrate reductase (30 mU), and 20 µl of ultrafiltrate sample in a 20 mM Tris-10 mM EDTA buffer, pH 7.4 mixture into a separate well in a white 96-well flat-bottom plate (Costar; Corning Glassworks, Corning, NY, USA). Samples were incubated for 90 min at 25°C. At the end of the incubation, 100 µl of freshly prepared sodium nitrite standard solutions (50–1000 pmol) were transferred into separate wells. This was followed by the addition of 30 µl of DAN solution (0.05 mg/ml in 0.62 N HCl) to each well and the plates were incubated in the dark for an additional 10 min. The reaction was stopped by adding 30 µl of 1.4 N NaOH. The intensity of the fluorescent signal was immediately measured using a fluorometer (FL600 Microplate Reader; Bio-Tek Instruments, Winooski, VT) at λ_{ex} = 360

and λ_{em} = 460. The concentration of NO_x in each sample was calculated from a standard curve generated by plotting the fluorescent units with a sodium nitrite concentration in picomoles using KC4 software (Bio-Tek Instruments). Results are expressed in picomoles per milligram of fresh tissue consistent with others (Seo and Revier, 2003). Total NO_x present in each sample was calculated as follows: total NO_x = [NO_x in sample (picomoles) x total volume added (microliters)/20 µl] per milligram of tissue.

Immunohistochemistry

For immunohistochemistry experiments, mice were anesthetized with chloral hydrate. Brains were fixed by cardiac perfusion with 1xPBS followed by 4% paraformaldehyde. After removal, brains were immersed in 4% paraformaldehyde overnight. The following morning, post-fixed brains were soaked in 20% sucrose until sectioning. Parasagital brain slices were obtained with a vibrating microtome. The selected slices were washed in 0.1% hydrogen peroxide for 30 min followed by blocking in 5% goat serum for 30 min. Then brain slices were probed overnight with the appropriate dilution of $\alpha_4\beta_2$ (1:1000) or α_7 (1:500) antibody. Subsequently, sections were washed three times with PBS followed by re-incubation with secondary antibody (1:5000) for 1-2 hours and again washing three times with PBS. Afterwards, sections were stained using a diaminobenzidine staining solution obtained as kit from Vector Laboratories (Burlingame, CA, USA). Immunoreactivity was examined with an Olympus BX51 microscope equipped with Normarski DIC optics under 12.5, 100 and 600x.

Statistical analysis

The Rotorod data were analyzed by analysis of variance (ANOVA) with two-way repeated measure in order to evaluate the effect of various drug doses and times on motor coordination. The drug dosage × time interaction effect was tested using the multivariate criterion of Wilk's lambda (λ). Significant drug × time interaction was evaluated using a one-way ANOVA. A Dunnett's C post hoc test was performed whenever significance was found on treatment and/or time. Statistical analyses were conducted using SPSS for Windows, version 16.0. Area under the curve (AUC) analysis was performed using GraphPad Prism 4.0©. Data comparing NO_x levels were analyzed by Student's t-test and one-way analysis of variance (ANOVA) and Bonferroni post hoc analysis. A p-value < 0.05 was taken as the level of significance in all statistical tests.

CHAPTER THREE: RESULTS

Consistent with previous work in this laboratory, ethanol produced profound ataxia that was measured starting at 15 min intervals post-ethanol injection time until the animals regained their normal motor coordination. Typically, ethanol control mice were able to walk on Rotorod at 15 min post-ethanol evaluation for only 40 sec; 65 sec at 30 min; 125 sec at 45 min. The animals generally regained their normal motor coordination (Rotorod walk for 180 sec) by 60 min post-ethanol injection.

Acute Intracerebellar (ICB) Microinfusion of RJR-2403 and acute Ethanol Ataxia: Functional Interaction

Effect of Intracerebellar Acute RJR-2403 on Ethanol Ataxia. Figure 8A demonstrates the dose-response relationship between the attenuating effect of various ICB doses of RJR-2403 on ethanol-induced ataxia. As shown in Fig. 8A, RJR-2403 markedly attenuated ethanol ataxia in a dose-related fashion. Significant drug treatment and time interaction $F_{(9,114)} = 22.54$, $p<0.05$ was observed. For ethanol control response ICB aCSF was microinfused in a group of animals instead of RJR-2403 followed by the administration of test dose of ethanol. All four RJR-2403 doses (31, 62, 125, 500 ng) produced statistically significant attenuation in ethanol ataxia compared to aCSF + ethanol control animals. Treatment with ICB RJR-2403 500ng caused total blockade of motor impairing effect of ethanol and abolished the ethanol ataxia. The reduction in ataxia following treatment with RJR-2403 at a dose of 125ng was 91% at 15 min ($p<0.05$) and 100% at 30, 45 and 60 min. Treatments with RJR-2403 62 ng and 31 ng

dose resulted in decrease in ethanol ataxia by 45% ($p<0.05$) and 21% ($p<0.05$) at 15 min and 83% ($p<0.05$) and 66% ($p<0.05$) at 30 min, respectively. Virtually, all animals in the RJR-2403 treated groups regained their normal motor coordination by 45 min with $p<0.05$ vs. ethanol controls. Figure 8B shows the area under the curve (AUC) analysis of the same treatment groups from Fig.8A. The AUC inversely correlates with the degree of ataxia observed after ICB drug treatment with RJR-2403 or aCSF. As the the dose of RJR-2403 increases from 31 to 500 ng, the AUC correspondingly decreases in a dose-related manner consistent with decreased cerebellar ethanol-induced ataxia. Thus, aCSF + ethanol (i.e. ethanol control group) exhibited the largest AUC and RJR-2403 500 ng + ethanol treatment the least. The specific drug treatments in Fig. 8A are indicated above the appropriate bars. There were no overt signs of CNS excitation due to any of the RJR-2403 doses selected in this study. When the highest dose of RJR-2403 (500 ng) was microinfused followed by saline instead of ethanol, it did not alter normal motor coordination. The EC_{50} of RJR-2403 was calculated to be 64.5 ng.

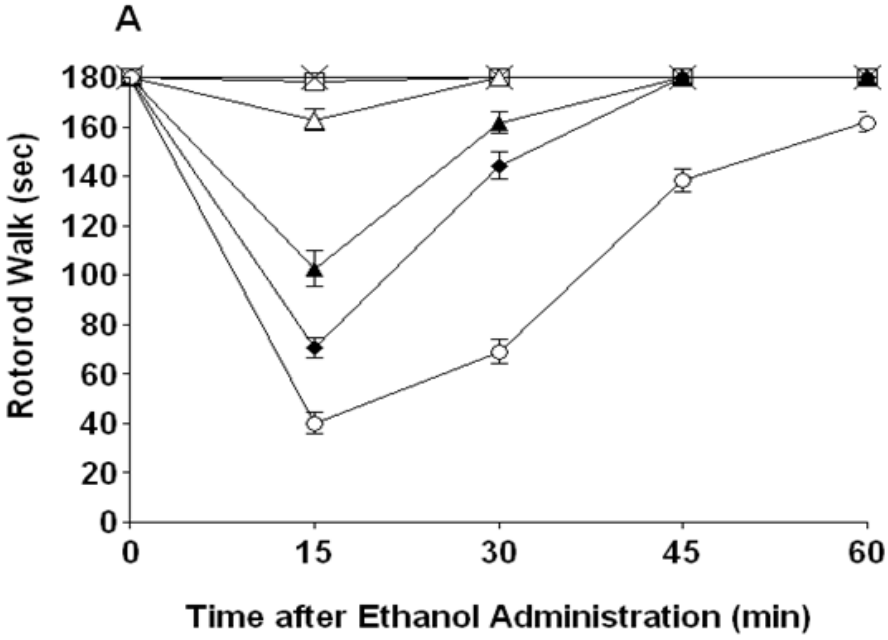
Effect of Acute Intracerebellar DHβE Pretreatment on RJR-2403-induced Attenuation of Ethanol Ataxia. Figure 9A shows that the attenuating effect of ICB RJR-2403 (125 ng) was markedly and dose dependently antagonized by ICB pretreatment with $\alpha_4\beta_2$-selective antagonist, DHβE. A significant drug treatment and time interaction $F_{(9,104)} = 10.52$, $p<0.05$ was observed. Treatment with a 500 ng dose of DHβE produced maximum and statistically significant antagonism of RJR-2403-induced attenuation of ethanol ataxia: 70% at 15 min, 50% at 30 min, 60% at 45 min with p value <0.05. DHβE (250 ng) treatment also produced marked reduction but only at 15 min (48%, $p<0.05$) and 30 min (30%, $p<0.05$). The smallest 125ng dose of DHβE resulted in a small but

significant antagonism at 15 min (20%, $p< 0.05$) only. The EC_{50} of RJR-2403 in the presence of DHβE was calculated to be 184.2 ng. When the order of DHβE and RJR-2403 administration was switched (i.e. RJR-2403 was microinfused 5 min prior to DHβE microinfusion), DHβE failed to antagonize RJR-2403's attenuating action because the animals displayed typical RJR-2403 responses (i.e. attenuation of ethanol ataxia). When ICB microinfusion of 500 ng dose of DHβE and RJR-2403 was followed by saline injection instead of ethanol, no alteration in the normal motor coordination was observed (Fig. 9A DHβE + RJR-2403 + saline, x---x). The data shown in Fig. 9A were also expressed as AUC values in Fig. 9B. The AUC analysis clearly demonstrates that with increasing doses of DHβE, RJR-2403-induced attenuation of ethanol ataxia was markedly reduced and consequently profound ataxia was observed. Thus, the higher the bar graph the greater was the degree of ataxia.

Effect of Acute Intracerebellar DHβE Pretreatment on Nicotine-Induced Attenuation of Ethanol Ataxia. To obtain further evidence regarding the role of $\alpha_4\beta_2$ subtype in the functional interaction between nicotine and ethanol, we investigated the effect of ICB pretreatment with DHβE on nicotine-induced attenuation of ethanol ataxia (Fig. 10A). A significant drug treatment and time interaction was observed [$F_{(9,104)} = 7.78$, $p<0.05$]. All doses (250, 500, 750 ng) of DHβE markedly and dose-dependently decreased nicotine's (5 ng) ability to reduce ethanol ataxia. DHβE (750 ng) treatment resulted in a significant reduction in nicotine-induced attenuating action at 15 (78%), 30 (75%) and 45 min (40%), respectively with $p<0.05$. Treatment with 500 ng DHβE produced 63%, 25% decrease in nicotine-induced attenuation of ethanol ataxia with $p<0.05$ at 15 and 30 min, respectively.

No change in nicotine-induced action at 45 min was noted as the animals maintained their normal motor incoordination. The smallest dose of DHβE (250 ng) produced a 34%

FIGURE 8: Effect of intracerebellar (ICB) microinfusion of various doses of $\alpha_4\beta_2$ nicotinic acetylcholine receptor subtype selective agonist, RJR-2403, on ethanol (2g/kg, i.p.)-induced ataxia. Each point represents the mean ± S.E.M. of 8-10 mice. **A**: ○ aCSF + ethanol; ♦ RJR-2403 31 ng + ethanol; ▲ RJR-2403 62 ng + ethanol; ∆ RJR-2403 125 ng + ethanol; □ RJR-2403 500 ng + ethanol; x RJR-2403 500ng + Saline. **B**: The AUC data are derived from the same treatment presented above in Fig. 8A; the treatments are shown above the appropriate bars.


A
Rotorod Walk (sec)
180
160
140
120
100
80
60
40
20
0
0
15
30
45
60
Time after Ethanol Administration (min)

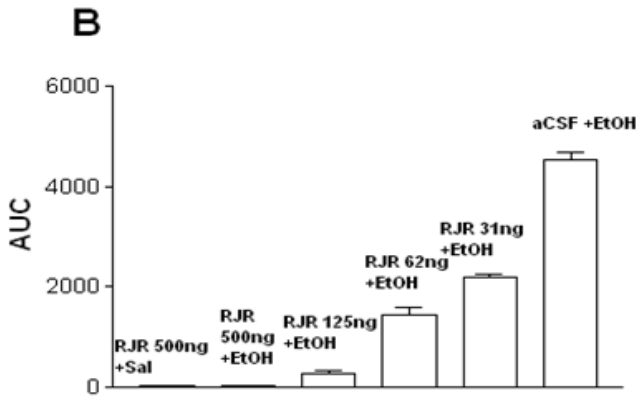
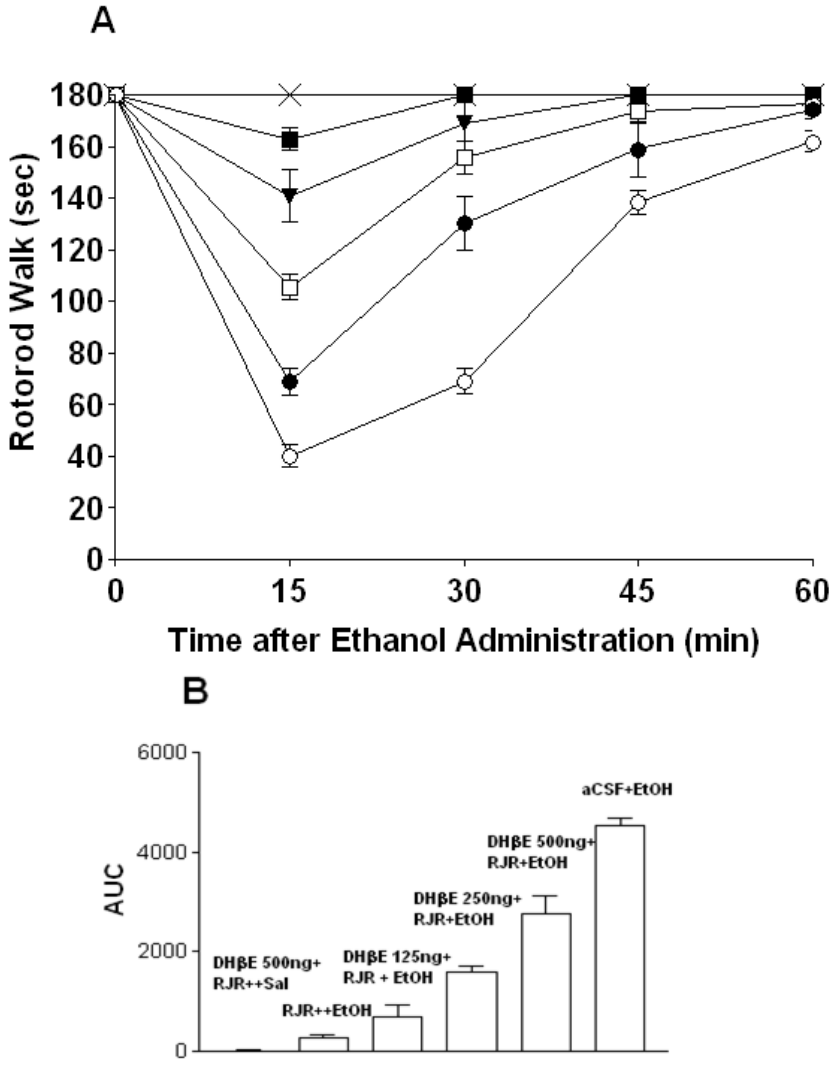

B
AUC
6000
4000
2000
0
RJR 500ng +Sal
RJR 500ng +EtOH
RJR 125ng +EtOH
RJR 62ng +EtOH
RJR 31ng +EtOH
aCSF +EtOH

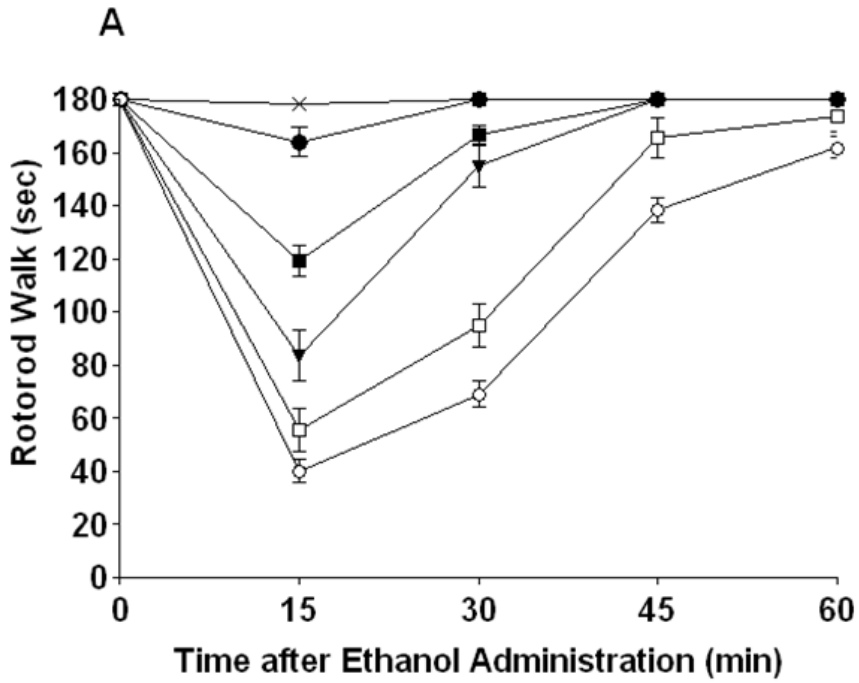
FIGURE 9: Effect of intracerebellar (ICB) microinfusion of various doses of the $\alpha_4\beta_2$-selective nicotinic acetylcholine receptor subtype antagonist, DHβE on RJR-2403 (125 ng; ICB)-induced attenuation of ethanol (2g/kg, i.p.) ataxia. Each point represents the mean ± S.E.M. of 8-10 mice. **A**: ○ aCSF + ethanol; ● DHβE 500 ng + RJR-2403 + ethanol; □ DHβE 250 ng + RJR-2403 + ethanol; ▼ DHβE 125 ng + RJR-2403 + ethanol; ■ RJR-2403 + ethanol; x DHβE 500 ng + RJR-2403 + Saline. **B**: The area under the curve (AUC) data are derived from the same treatments presented above; the treatments are shown above the appropriate bars.


A
Rotorod Walk (sec)
180
160
140
120
100
80
60
40
20
0
0
15
30
45
60
Time after Ethanol Administration (min)
B
AUC
6000
4000
2000
0
aCSF+EtOH
DHβE 500ng+
RJR+EtOH
DHβE 250ng+
RJR+EtOH
DHβE 125ng+
RJR + EtOH
DHβE 500ng+
RJR++Sal
RJR++EtOH

($p<0.05$) and 15% decrease in nicotine's attenuating action at 15 and 30 min, respectively. There was no change in nicotine-induced attenuation at 45 min post-ethanol administration as the animals maintained their normal motor coordination. DHβE was shown to be slightly more potent in antagonizing RJR-2403's action than nicotine's. In Fig.10B, the same data which was presented in Fig. 10A have been expressed as AUC. DHβE pretreatment dose-dependently increased ethanol ataxia due to antagonism of nicotine-induced attenuation of ethanol ataxia. As such, the higher the dose of DHβE, the greater was the ataxia and the height of the bar graph (Fig. 10B).

Effect of α_4 Oligonucleotide treatment on RJR-induced attenuation of Ethanol Ataxia As shown in Fig. 11A pretreatment with α_4 nAChR subtype antisense (200nM, twice a day) significantly ($p<0.05$) blocked and virtually abolished RJR-2403-induced attenuation of ethanol ataxia. The ability of RJR-2403 to attenuate ethanol-induced ataxia was diminished by 54% at 15 min post-ethanol ($p<0.05$) compared to missense treated group (Fig.11A. ■---■ vs.▲---▲) and by 74% ($p<0.05$) compared to RJR-2403+ethanol group (Fig. 11A. ■---■ vs. □---□). There was a significant drug treatment and time interaction $F_{(6, 72)} =12.67$, $p<0.05$. Also, at subsequent times (i.e. 30 and 45 min) there was a significant ($p<0.05$) impairment in the ability of RJR-2403 to attenuate ethanol ataxia due to antisense treatment versus that of missense. However, antisense treated mice regained their normal motor coordination relatively quickly compared to aCSF + ethanol treated group. Figure 11B shows the expression of Rotorod data (Fig. 11A) as AUC.

FIGURE 10: Effect of intracerebellar (ICB) microinfusion of various doses of a selective antagonist of $\alpha_4\beta_2$ nicotinic acetylcholine receptor subtype, DHβE on (-)-nicotine (5 ng; ICB)-induced attenuation of ethanol (2g/kg, i.p.) ataxia. Each point represents the mean ± S.E.M. of 8-10 mice. **A**: ○ aCSF + ethanol; □ DHβE 750 ng + (-)-nicotine + ethanol; ▼ DHβE 500 ng + (-)-nicotine + ethanol; ■ DHβE 250 ng + (-)-nicotine + ethanol; ● (-)-nicotine + ethanol; x DHβE 500 ng + (-)-nicotine + Saline. **B**: The area under the curve (AUC) data are derived from the same treatments presented above in A; the treatments are shown above the appropriate bars.


A
Rotorod Walk (sec)
180
160
140
120
100
80
60
40
20
0
0
15
30
45
60
Time after Ethanol Administration (min)

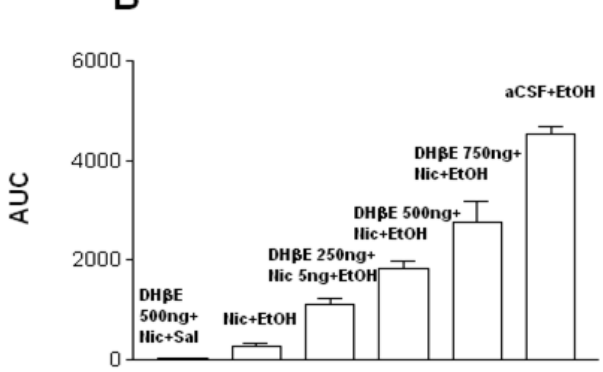
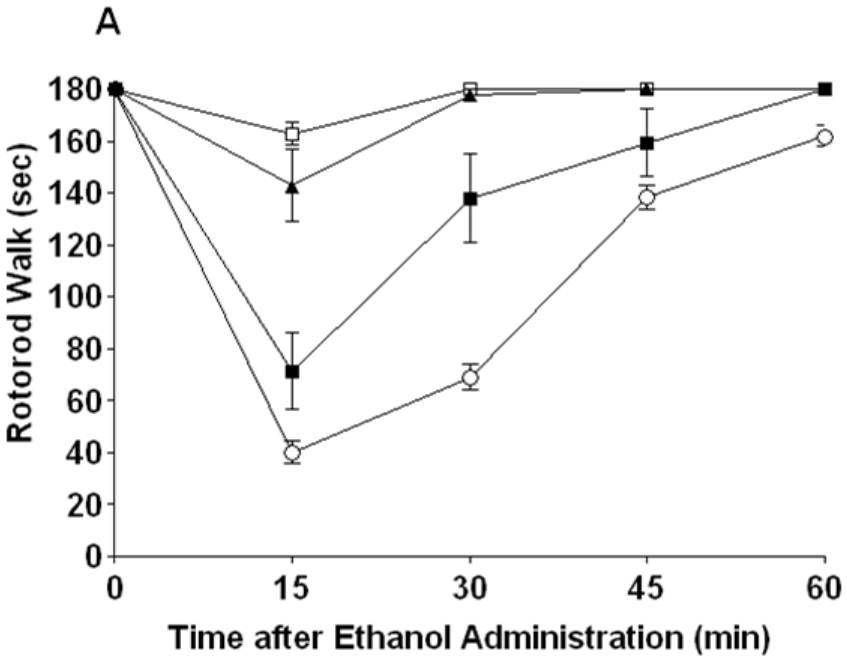
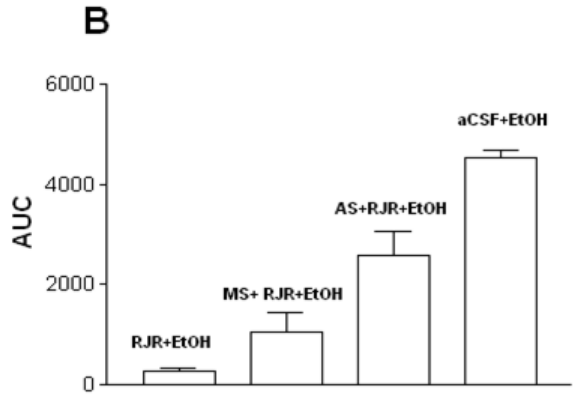

B
AUC
6000
4000
2000
0
DHβE 500ng+ Nic+Sal
Nic+EtOH
DHβE 250ng+ Nic 5ng+EtOH
DHβE 500ng+ Nic+EtOH
DHβE 750ng+ Nic+EtOH
aCSF+EtOH

FIGURE 11: Effect of repeated intracerebellar (ICB) microinfusion of antisense and missense (200nM; twice daily for 4 days) selective for the α_4 nicotinic acetylcholine receptor subtype on RJR-2403 (500ng, ICB)-induced attenuation of ethanol (2g/kg, i.p.) ataxia. Each point represents the mean ± S.E.M. of 10-12 animals. **A:** ○ aCSF + ethanol; ■ Antisense + RJR-2403 + ethanol (n=10); ▲Missense x 4days + ethanol (n=6); □ RJR-2403 + ethanol (n=12). **B:** The area under the curve (AUC) data are derived from the same treatments presented above in **A;** the treatments are shown above the appropriate bars.


A
Rotorod Walk (sec)
180
160
140
120
100
80
60
40
20
0
0
15
30
45
60
Time after Ethanol Administration (min)


B
AUC
6000
4000
2000
0
RJR+EtOH
MS+ RJR+EtOH
AS+RJR+EtOH
aCSF+EtOH

Western Blot Experiment

For comparison of α_4 nAChR expression in antisense and missense treated mice immunoblot experiments were performed on cerebellar tissue (Fig. 12A). According to our data, in contrast to the missense oligonucleotides, antisense oligonucleotides decreased α_4 protein expression markedly but did not abolish it completely as a faint protein band was visible at ~ 70kd in the antisense treatment sample. This was consistent with the Rotorod data (Fig. 12A) and may explain the relatively faster recovery of antisense treated animals at 30 and 45 min compared to aCSF + ethanol treated group (Fig. 12A ■---■ vs. □---□). The peak band intensity in the missense and the antisense sample was 84.33% and 50.48%, respectively (Fig. 12B). GAPDH loading controls depict quantitatively similar protein abundance in each lane of the SDS-PAGE-gel.

Histology

Histological examination of coronal sections of cerebellum with cresyl violet stain shows no morphological alteration or tissue damage following 4 days of antisense and missense treatment (Figs. 13A and B).

Acute Intracerebellar (ICB) microinfusion of PNU-282987 and Ethanol Ataxia: Functional Interaction

Effect of Acute Intracerebellar PNU-282987 on Ethanol Ataxia. As shown in Fig. 14A, ICB administration of PNU-282987 caused almost dose-dependent attenuation of ethanol ataxia. Three different doses of PNU-282987, 25ng, 250 ng, 2.5µg, were used in order to establish a dose-response relationship between PNU-282987 and the attenuation of the ethanol ataxia. A significant (77% and 73%; $p<0.05$) reduction in

FIGURE 12: Western blot showing the significant reduction of α_4-subunit expression following antisense treatment versus missense treatment (A). The lower panel depicts equal loading of the samples by expressing the identical levels of GAPDH protein. The relative peak density of corresponding bands is shown in Fig. 12B.

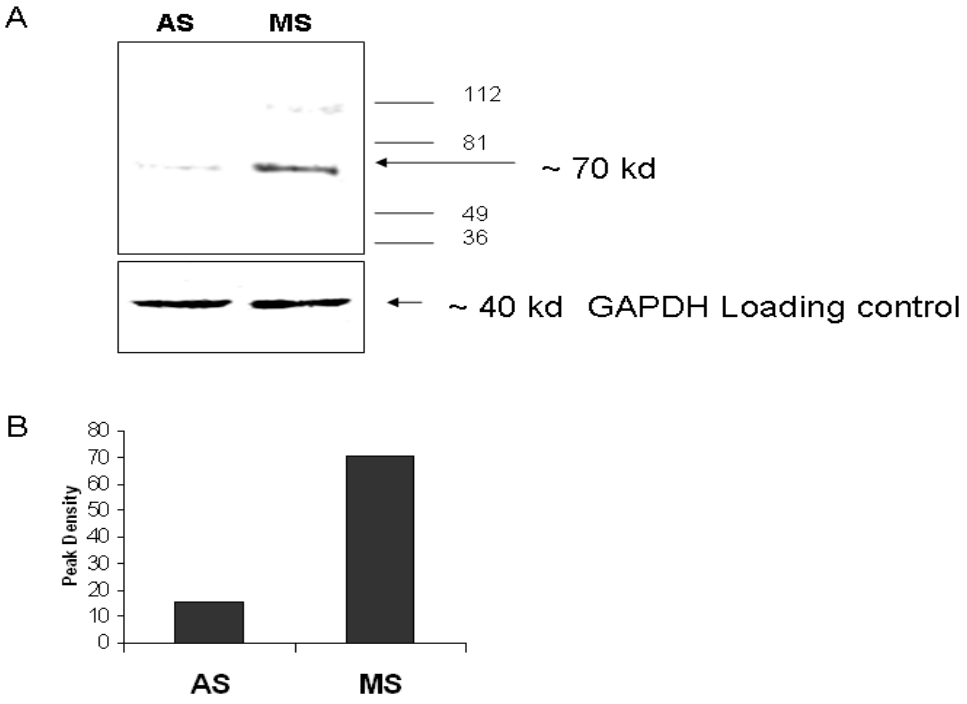
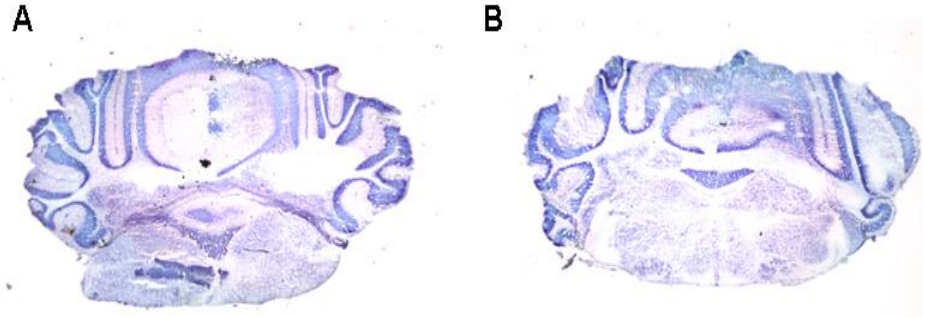

A
AS
MS
112
81
~ 70 kd
49
36
~ 40 kd GAPDH Loading control
B
Peak Density
80
70
60
50
40
30
20
10
0
AS
MS

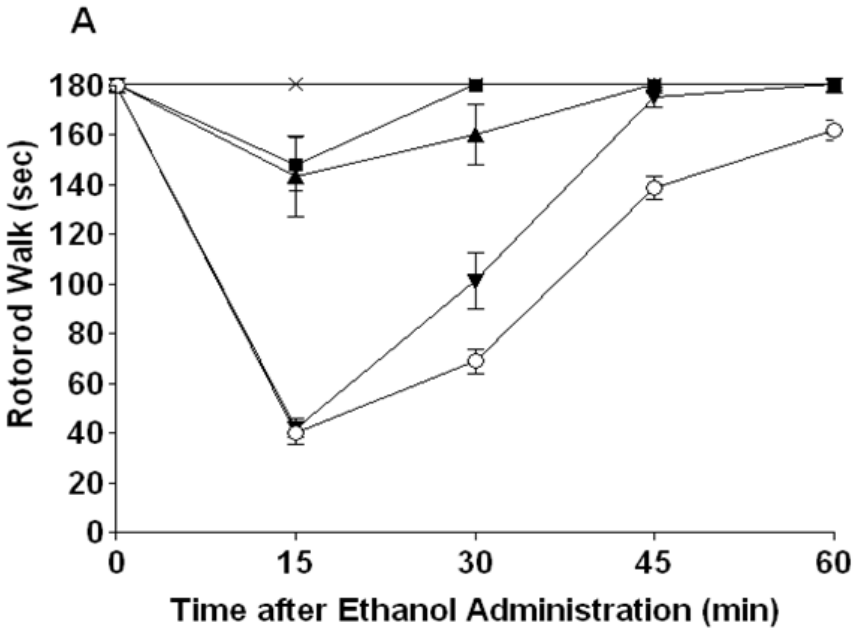
FIGURE 13: Histological photomicrograph of coronal sections of the cerebellum of mice treated with missense (A) and antisense (B).

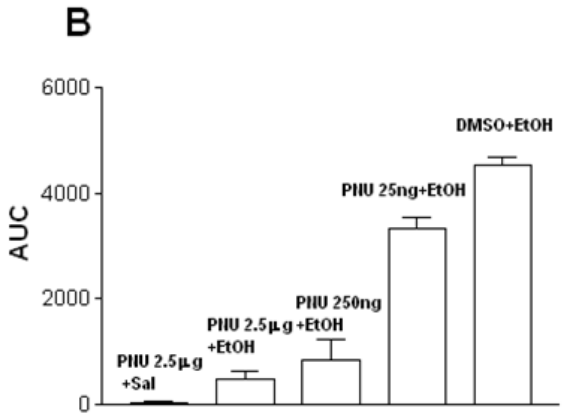

A
B

ethanol ataxia at 15 min post-ethanol injection, following microinfusion of 250ng and 2.5µg dose of PNU-282987, respectively, was observed. At subsequent post-ethanol Rotorod evaluation times (30, 45, 60 min), animals exhibited nearly 100% normal motor coordination (i.e., walked 180 seconds on the Rotorod). The lowest (25ng) PNU-282987 dose did not produce any effect on ethanol ataxia at 15 min, however, at the other three motor evaluation periods, a modest but pronounced ($p<0.05$; 33%, 100%, 100%, respectively) reduction in ataxia was observed. Overall, a significant drug treatment and time interaction was observed ($F_{(12,132)} =11.56$, $p<0.001$). Figure 14B. represents the area under the curve (AUC) data of the same treatment groups as presented in Fig. 14A. The AUC correlates well with the degree of ethanol ataxia: the greater the AUC of a treatment group, the higher was the degree of ataxia noted. Hence, the AUC for the PNU-282987-treated group decreased with the increasing dose of PNU-282987. The PNU-282987 25ng + ethanol and aCSF + ethanol control groups exhibited the highest AUC.

Effect of Intracerebellar Methyllycaconitine Pretreatment on PNU-282987-Induced Attenuation of Ethanol Ataxia. To verify that PNU-282987-induced attenuation of ethanol ataxia was mediated through the α_7 nAChR subtype, mice were pretreated with ICB methyllycaconitine, an α_7- subtype selective antagonist, followed by ICB PNU-282987 and systemic ethanol. Figure 15A shows that the effect of a 6 ng dose of methyllycaconitine was significant on PNU-282987-induced attenuation of ethanol ataxia. The drug treatment and time interaction was found to be significant ($F_{(9,141)} = 10.612$, $p<0.001$). Pretreatment with methyllycaconitine completely abolished the effect of PNU-282987 on ethanol ataxia at all four motor evaluation time periods ($p<0.05$). Thus, the

FIGURE 14: The effect of intracerebellar (ICB) microinfusion of α_7-subtype selective nicotinic acetylcholine receptor agonist, PNU-282987, on ethanol (2g/kg, i.p.)-induced ataxia. Each point represents the mean ± S.E.M. of 10 mice. **A:** ○ aCSF + ethanol; ▼ PNU 25 ng + ethanol; ▲ PNU 250 ng + ethanol; ■ PNU 2.5 µg + ethanol; x PNU 2.5 µg + Saline. **B:** The area under the curve (AUC) data are derived from the same treatments presented above in **A;** the treatments are shown above the appropriate bars.


A
Rotorod Walk (sec)
180
160
140
120
100
80
60
40
20
0
0
15
30
45
60
Time after Ethanol Administration (min)


B
AUC
6000
4000
2000
0
PNU 2.5µg +Sal
PNU 2.5µg +EtOH
PNU 250ng
PNU 25ng+EtOH
DMSO+EtOH

dose response curve for methyllycaconatine + PNU + ethanol treatment was comparable to that of aCSF + ethanol control group. However, the methyllycaconitine + PNU + saline treatment group did not display any alteration in the normal motor coordination. The evaluation of AUC data shows an inverse relationship with the degree of ataxia (Fig. 15B). Consequently, the AUC of methyllycaconitine + PNU + ethanol (Fig. 15A ♦---♦) treatment was markedly greater (i.e., maximum ataxia) compared to PNU2.5µg + ethanol (Fig. 15A ∆---∆) or methyllycaconitine + PNU + saline (Fig. 15A x---x) treatments.

Effect of Acute Intracerebellar Methyllycaconitine Pretreatment on RJR-2403-Induced Attenuation of Ethanol Ataxia. To confirm the selectivity of α_7 selective agonist and antagonist employed, mice were pretreated with 12 or 6 ng dose of α_7-subtype selective antagonist, methyllycaconitine, followed by the administration of $\alpha_2\beta_4$-selective agonist RJR-2403 (ICB 125ng; Fig. 16A). Pretreatment with the highest (12ng) dose of methyllycaconitine was able to partially block (51% with $p<0.05$) RJR-induced attenuation of ethanol-ataxia suggesting that at this high dose, methyllycaconitine lost its α_7-selectivity and additionally antagonized the $\alpha_4\beta_2$ nAChR subtype (Fig. 16A; ▲---▲). At 6ng dose, methyllycaconitine did not alter RJR-2403-induced attenuating action while the same dose totally abolished the action of PNU-282987 indicating selective α_7 subtype nAChR blockade at this dose (Fig. 15A). A significant drug treatment and time interaction was observed [$F_{(9,119)} = 17.95$, $p<0.05$]. Figure 16B summarizes the AUC for each treatment group used in Fig. 16A, as the degree of ethanol ataxia increased, the AUC decreases and vice versa.

FIGURE 15: The effect of pretreatment on intracerebellar (ICB) microinfusion of the α_7-subtype selective nicotinic acetylcholine receptor antagonist, methyllycaconitine (MLA; 6ng) on PNU (2.5µg)-induced attenuation of ethanol (2g/kg, i.p.) ataxia. Each point represents the mean ± S.E.M. of 10 mice. **A:** ○ aCSF + ethanol; ♦ MLA 6 ng + PNU 2.5µg + ethanol; ∆ PNU 2.5 µg + ethanol; x MLA 6 ng + PNU 2.5 µg + Saline. **B:** The area under the curve (AUC) data are derived from the same treatment presented above in **A;** the treatments are shown above the appropriate bars.

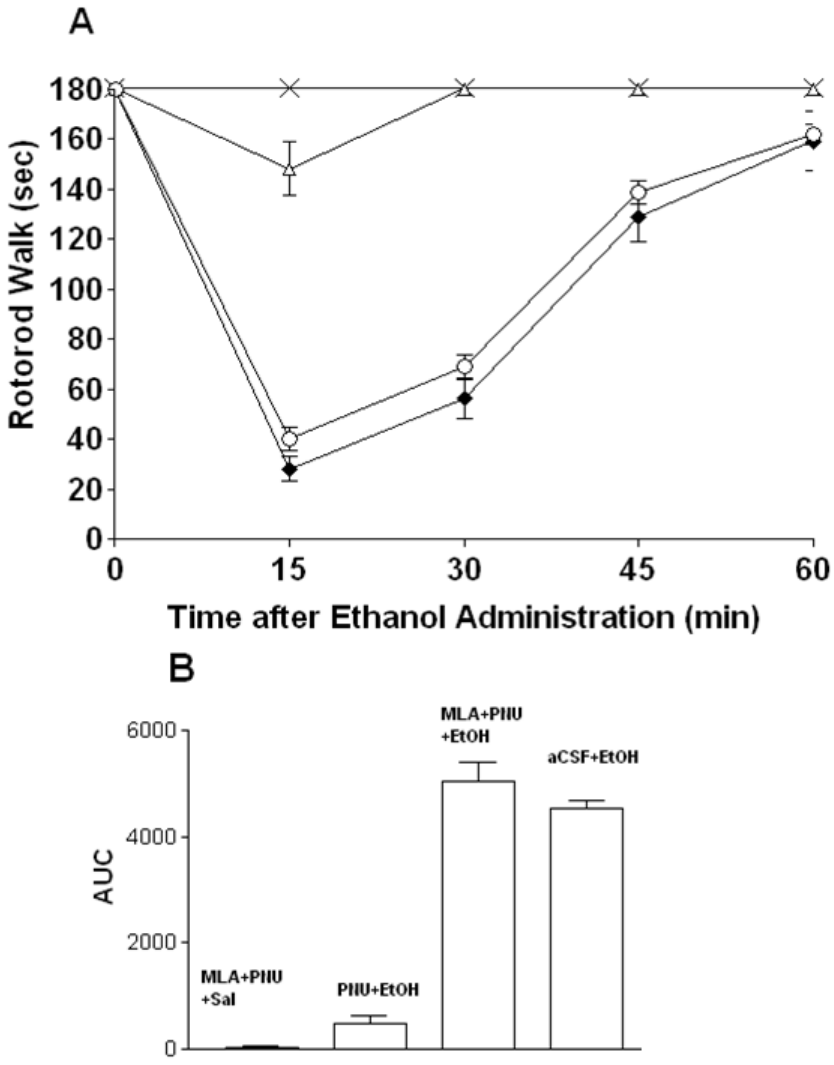
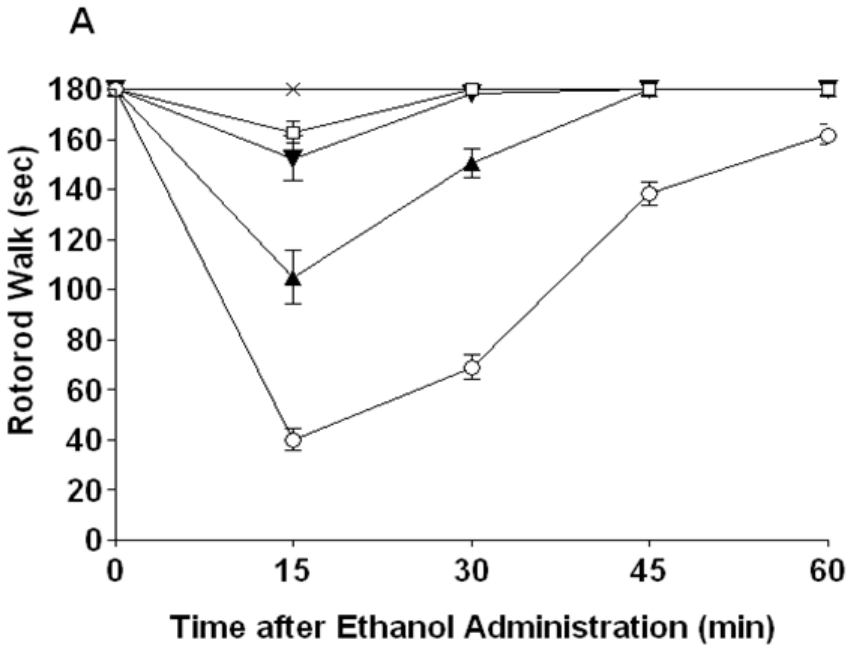
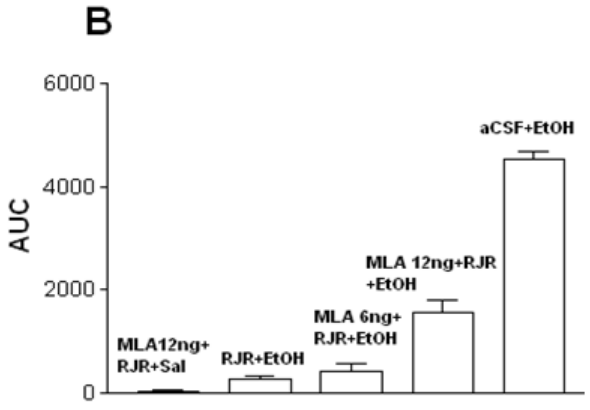

A
Rotorod Walk (sec)
180
160
140
120
100
80
60
40
20
0
0
15
30
45
60
Time after Ethanol Administration (min)
B
AUC
6000
4000
2000
0
MLA+PNU
+Sal
PNU+EtOH
MLA+PNU
+EtOH
aCSF+EtOH

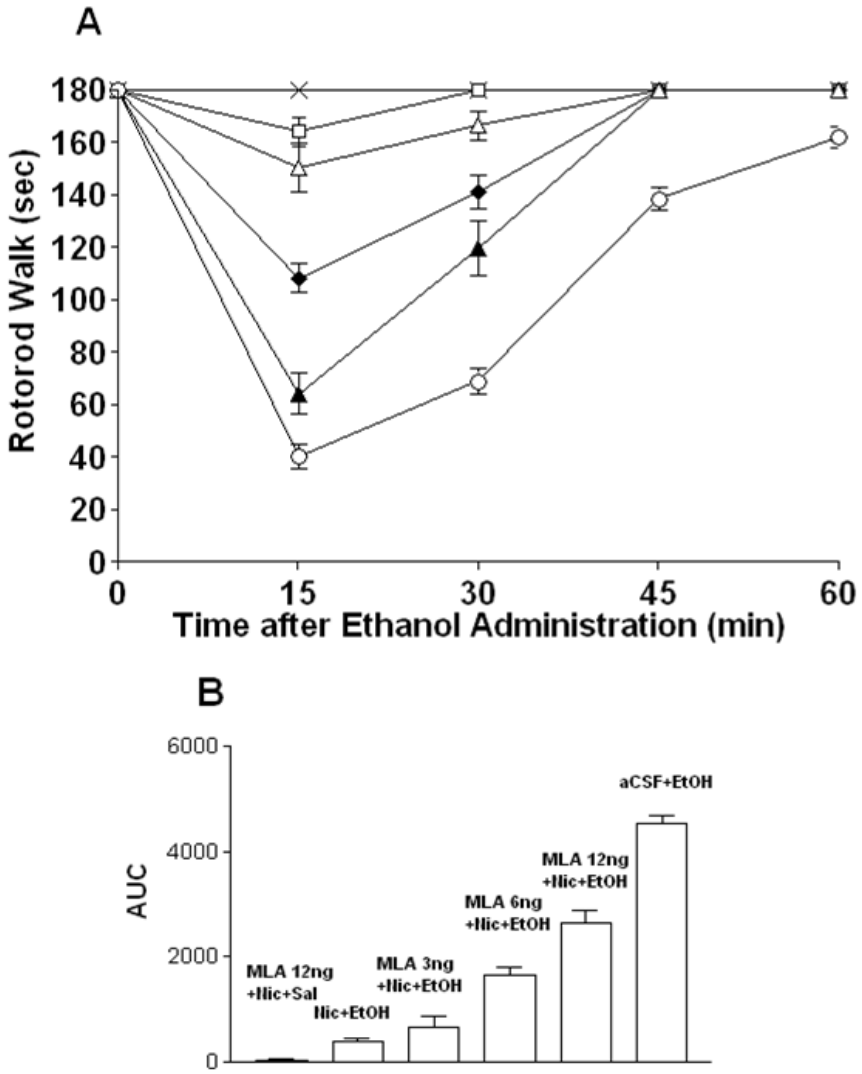
FIGURE 16: The effect of intracerebellar (ICB) microinfusion of various doses of a selective antagonist at the α_7 nicotinic acetylcholine receptor subtype, methyllycaconitine (MLA) on RJR-2403 (125ng, ICB), a selective agonist at $\alpha_4\beta_2$ nicotinic acetylcholine receptor subtype-induced attenuation of ethanol (2g/kg, i.p.) ataxia. Each point represents the mean ± SEM of 10 mice. **A:** ○ aCSF + ethanol; ▲ MLA 12 ng + RJR + ethanol; ▼ MLA 6 ng + RJR + ethanol; □ RJR + ethanol; x MLA 12ng + RJR + Saline. **B:** The area under the curve (AUC) data are derived from the same treatments presented above in Fig. 16A; the treatments are shown above the appropriate bars.


A
Rotorod Walk (sec)
180
160
140
120
100
80
60
40
20
0
0
15
30
45
60
Time after Ethanol Administration (min)


B
AUC
6000
4000
2000
0
MLA12ng+
RJR+Sal
RJR+EtOH
MLA 6ng+
RJR+EtOH
MLA 12ng+RJR
+EtOH
aCSF+EtOH

Effect of Intracerebellar Methyllycaconitine Pretreatment on Nicotine-Induced Attenuation of Ethanol Ataxia. To further strengthen our findings presented in Figs. 14 and 15, and to provide possible evidence that nicotine-induced attenuation of ethanol ataxia as reported previously (Al-Rejaie and Dar, 2006a,b,c) is mediated, at least in part, through the α_7 nAChR subtype, methyllycaconitine was microinfused as pretreatment prior to ICB nicotine administration. Figure 17A shows the effect of three different doses (3, 6, 12ng) of methyllycaconitine on nicotine-induced attenuation of ethanol ataxia. There was a dose-dependent reduction in nicotine-induced attenuation of ethanol ataxia caused by methyllycaconitine pretreatment. A significant drug treatment and time interaction [$F_{(15,182)}$ = 14.01, $p<0.001$] was observed. The highest dose (12ng) of methyllycaconitine used in the present study caused a 74% and 58% ($p<0.05$) decrease in nicotine-induced attenuation at 15 and 30 min, respectively (Fig.17A ;▲---▲ vs. □---□). The methyllycaconitine dose of 6 ng produced a 41% and 33% ($p<0.05$). decrease in nicotine-induced attenuation of ethanol ataxia at 15 and 30 min, respectively (Fig. 17A; ♦---♦ vs. □---□). The 3ng dose of methyllycaconitine lacked any significant effect on nicotine's ability to attenuate ethanol ataxia (Fig. 17A: ∆---∆ vs. □---□). When the highest dose of methyllycaconitine was microinfused followed by ICB microinfusion of nicotine and saline injection instead of ethanol, there was no change in the normal motor coordination, thus precluding the possibility of tonic involvement of the α_7 subtype in the normal coordination. It also may suggest selectivity of ethanol in its functional interaction with nicotine. Therefore, microinfusion of methyllycaconitine + nicotine + saline plays no role in the normal motor coordination. Likewise, in Fig. 17B, the AUC of methyllycaconitine + nicotine + ethanol treatment group increases with increasing doses

FIGURE 17: The effect of intracerebellar microinfusion of various doses of, methyllycaconitine (MLA), a selective antagonist at the α_7 nicotinic acetylcholine receptor subtype, on (-)-nicotine (5ng, ICB)-induced attenuation of ethanol (2g/kg, i.p.) ataxia. Each point represents the mean $\pm$ S.E.M. of 10 mice. **A:** ○ aCSF + ethanol; ▲ MLA 12 ng + (-)-nicotine + ethanol; ♦ MLA 6 ng + (-)-nicotine + ethanol; ∆ MLA 3 ng + (-)-nicotine + ethanol; □ (-)-nicotine + ethanol; x MLA 12 ng + (-)-nicotine + Saline. **B:** The area under the curve (AUC) data are derived from the same treatments presented above in **A;** the treatments are shown above the appropriate bars.


A
Rotorod Walk (sec)
180
160
140
120
100
80
60
40
20
0
0
15
30
45
60
Time after Ethanol Administration (min)
B
AUC
6000
4000
2000
0
MLA 12ng
+Hic+Sal
Hic+EtOH
MLA 3ng
+Hic+EtOH
MLA 6ng
+Hic+EtOH
MLA 12ng
+Hic+EtOH
aCSF+EtOH

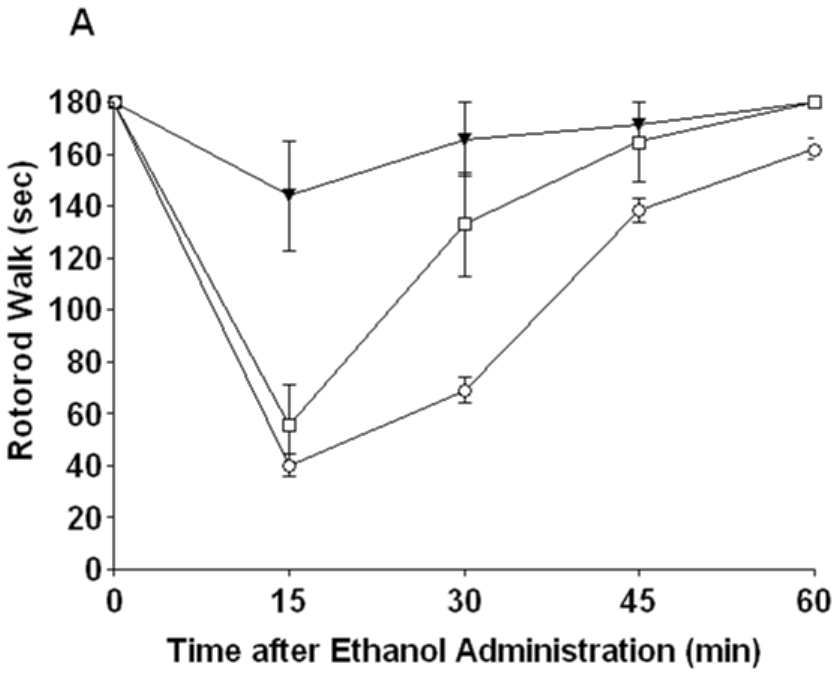
of methyllycaconitine to denote occurrence of profound ataxia associated with nullification of nicotine-induced attenuation of ethanol ataxia. On the other hand, nicotine + ethanol treatment virtually abolished the ethanol ataxia which correlated with reduced AUC.

Effect of α_7 Oligonucleotide treatment on PNU-induced attenuation of Ethanol Ataxia Figure 18A, illustrates the effect of ICB repeated antisense and missense oligonucleotide treatment on PNU-282987-induced attenuation of ethanol ataxia. The antisense against the α_7 nAChR subtype significantly reduced the ability of PNU-282987 to attenuate ethanol ataxia compared to missense treated control (Fig. 18A; □--□ vs. ▼---▼). The effect of the repeated ICB microinfusion of the α_7 -subtype antisense was maximal at 15 min post-ethanol administration because the degree of ataxia was comparable between the antisense-pretreated animals that received PNU-282987 + ethanol treatment and the aCSF + ethanol treated group (Fig. 18A: □--□ vs. ○---○). Overall, there was significant drug treatment and time interaction ($F_{(9,119)}$ =17.95, $p<0.05$). Figure 18B shows the AUC of the same treatment groups as in Fig. 18A. The overall AUC of the antisense + PNU-282987+ethanol treated mice was higher than that of missense + PNU-282987+ethanol-treated control groups.

Western Blot Data

Western blot analysis carried out on cerebellar cortical tissue samples obtained from missense control and antisense-treated mice failed to show a significant difference between two groups indicating that antisense may have a transient effect on the reduction of α_7 expression at the cerebellar cortical microinfusion site (Fig. 19).

FIGURE 18: The effect of chronic intracerebellar (ICB) microinfusion of antisense (ICB, 200nM; b.i.d. x 4 days) selective for α_7 nicotinic acetylcholine receptor subtype and missense on PNU-282987 (250ng, ICB)-induced attenuation of ethanol (2g/kg, i.p.) ataxia. Each point represents the mean ± S.E.M. of 10 animals. **A:** ○ aCSF + ethanol; □ antisense 200nM b.i.d. x 4 days + PNU + ethanol (n=10); ▼ missense + PNU + ethanol (n=12). **B:** The area under the curve (AUC) data are derived from the same treatment presented above in **A** the treatments are shown above the appropriate bars.


A
Rotorod Walk (sec)
180
160
140
120
100
80
60
40
20
0
0
15
30
45
60
Time after Ethanol Administration (min)

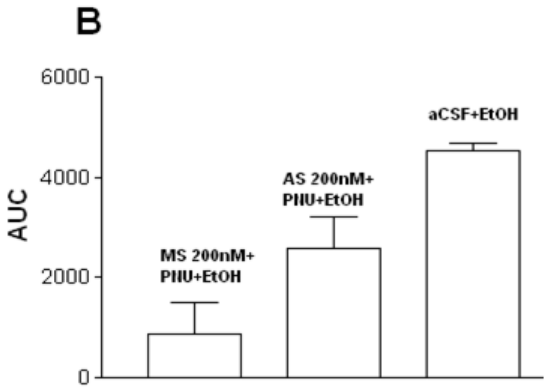
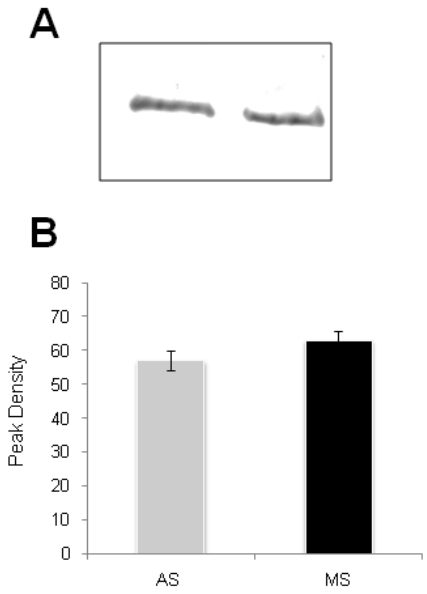

B
AUC
6000
4000
2000
0
MS 200nM+
PNU+EtOH
AS 200nM+
PNU+EtOH
aCSF+EtOH

FIGURE 19: A: Western blot results showing the levels of α_7 expression following antisense treatment versus missense control treatment. B: The relative peak density of corresponding bands as shown in Fig. 19A.


A
B
Peak Density
80
70
60
50
40
30
20
10
0
AS
MS

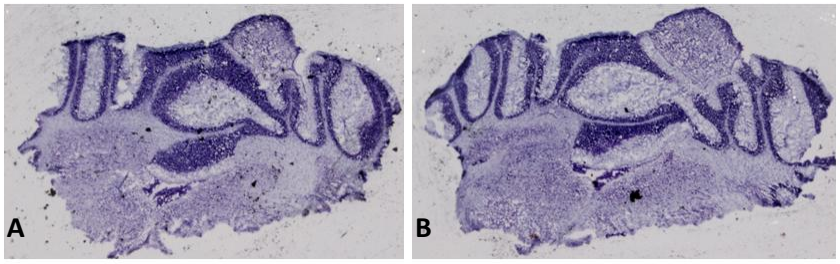
Histology

Histological examination of coronal sections of cerebellum with cresyl violet stain shows no morphological alteration or tissue damage following 4 days of antisense and missense treatment (Figs. 20A and B).

Repeated Intracerebellar Microinfusion of RJR-2403 and Ethanol Ataxia: Development of Cross-Tolerance

Dose-Response Relationship between Repeated Intracerebellar RJR-2403 and Acute Ethanol Ataxia. Figure 21A demonstrates the relationship between repeated (once daily for 5 days) ICB microinfusion of various doses of RJR-2403, 16 hr after the last RJR-2403 microinfusion, on acute ethanol (2g/kg; i.p)-induced motor incoordination. As shown in Fig. 21A, RJR-2403 (31, 62 and 125ng) treatment attenuated acute ethanol ataxia nearly in a dose-dependent manner. Acute ethanol was administered 16h after the last RJR-2403 microinfusion. Animals in the control group received aCSF instead of RJR-2403. The 125 ng dose of RJR-2403 produced the largest and statistically significant ($p < 0.01$: 57%) reduction in ethanol ataxia versus ethanol controls at 15 min post-ethanol injection (Fig. 21A: ♦--♦ vs. ○---○). For subsequent tor evaluation times, there was no ataxia observed because all animals regained their normal motor coordination (Fig. 21A: ♦--♦ vs. ○---○). There was no significant difference between the attenuation response of 31ng and 62ng doses of RJR-2403 at various evaluation time points. Only RJR-2403 (62ng) produced a significant 21% ($p<0.05$) reduction in ethanol ataxia at 15 min. Overall, a significant drug treatment and time interaction was observed ($F_{9,107} = 4.43$; $p < 0.001$). Repeated ICB pretreatment with DHβE infused 15 min before

FIGURE 20: Histological photomicrograph of coronal sections of cerebellum of mice treated with missense control (A) and antisense **(B)**


A
B

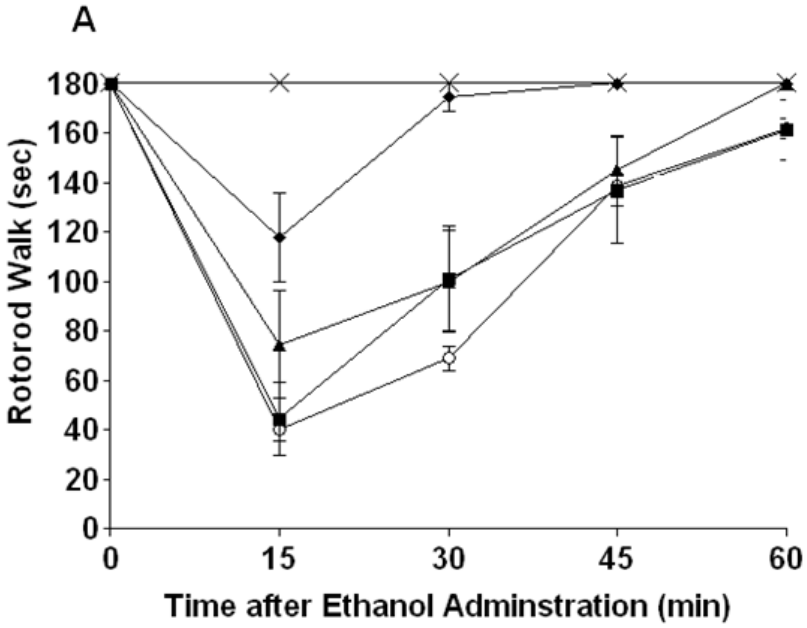
daily RJR-2403 treatment virtually abolished the development of cross-tolerance between RJR-2403 (125ng) and acute ethanol ataxia. As also shown in Fig. 21A, RJR-2403 (125ng; once daily for 5 days) + saline treatment did not alter normal motor coordination. Figure 21B represents AUC data for the same treatment groups presented in Fig. 21A. There is a direct relationship between AUC and the degree of ataxia i.e., the greater the ataxia the larger is the bar size. Therefore, AUC is largest for aCSF + ethanol and RJR-2403 31ng + ethanol treatments but relatively small for both the higher doses (125 and 62ng) of RJR-2403 + ethanol.

Initiation Time for Development of Cross-Tolerance Between Repeated Intracerebellar RJR-2403 And Acute Ethanol Ataxia. To determine the time required for the onset of cross-tolerance between repeated RJR-2403 and acute ethanol ataxia, RJR-2403 was microinfused for 1, 2, 3, 5, 7 days (once daily) followed 16 h later after the last RJR-2403 treatment by acute ethanol administration and Rotorod evaluation. The RJR-2403 dose of 125 ng was selected in these experiments based on previously conducted dose response study (Fig. 21A). As shown in Fig. 22A, the result of RJR-2403 treatment for 2, 3, and 5 days was a significant attenuation of acute ethanol ataxia that indicated the development of cross-tolerance at all four Rotorod evaluation time periods. There was a significant drug treatment and time interaction ($F_{18, 200} = 3.44$; $p<0.001$). The reduction in ethanol ataxia in the 2, 3, and 5-day RJR-2403 treatment groups was as follows: 2-day (30%), 3-day (55%), 5-day (57%) with $p<0.01$ versus control at 15 min post-ethanol administration (Fig. 22A: ♦---♦; ▲---▲; ■---■ vs. ○---○). At 30, 45, and 60 min post-ethanol evaluation times the animals virtually regained their normal motor coordination. There was no significant alteration in ethanol ataxia 16h after single RJR-2403 treatment (1-day) at

15 min but significant attenuation at 30, 45 and 60 min post-ethanol was observed (Fig. 22A: □---□ vs. ▼---▼). When RJR was microinfused for 7 consecutive days, there was not much difference in the results of attenuation of ethanol ataxia between RJR-2403 1-day and 7-day treatment groups. In the control group in which, RJR-2403 was microinfused for 5 days followed by saline injection instead of ethanol, no change in the normal motor coordination was noted (x---x vs. ○---○) .Figure 22B shows the results of the same treatment groups that were used in Fig. 22A expressed as AUC. As already stated above, the AUC displays a direct relationship with the degree of ataxia. The optimum time for the development of cross-tolerance was observed following microinfusion of RJR-2403 (once daily) for 5-day and not in 7-day RJR-2403 treatment group.

Duration of Cross-Tolerance Between Intracerebellar RJR-2403 and Acute Ethanol Ataxia. Figure 23 shows the duration of time for which the cross-tolerance between RJR-2403 and ethanol ataxia remained observable. Similar to the study for the onset of the development of cross-tolerance (Fig. 22), the ICB treatment with RJR-2403 (125 ng; once daily for 5 days) was the same. The animals were challenged with the same test dose of acute ethanol at 36, 48 and 72 h after the last ICB microinfusion of RJR-2403. The results indicate that the cross-tolerance lasted nearly 48 h from the last RJR administration because at 72 h post-RJR-2403 infusion, no cross-tolerance was apparent. The Rotorod response curve for 72h group overlapped the ethanol control group (Fig. 23A: *----* vs. ○---○) indicating a complete loss of cross-tolerance. The cross-tolerance between repeated RJR-2403 and ethanol ataxia developed maximally at 16h from the last RJR-2403 microinfusion in a 5-day treatment group: 57% ($p<0.05$)

Figure. 21. The effect of repeated intracerebellar (ICB) microinfusion of RJR-2403 (31, 62 or 125ng; once daily for 5 days), 16 hrs following the last intracerebellar RJR microinfusion, on acute ethanol (2g/kg, ip)-induced ataxia. Each point represents the mean ± S.E.M of 10 mice. **A:** ○ aCSF + Ethanol (2g/kg,i.p.); ■ RJR (31ng) + Ethanol; ▲ RJR (62ng) + Ethanol; ♦ RJR (125ng) + Ethanol; × RJR (125ng) + Saline. **B:** The area under the curve (AUC) data are derived from the same treatments presented above in **A;** the treatments are shown above the appropriate bars.


A
Rotorod Walk (sec)
180
160
140
120
100
80
60
40
20
0
0
15
30
45
60
Time after Ethanol Adminstration (min)

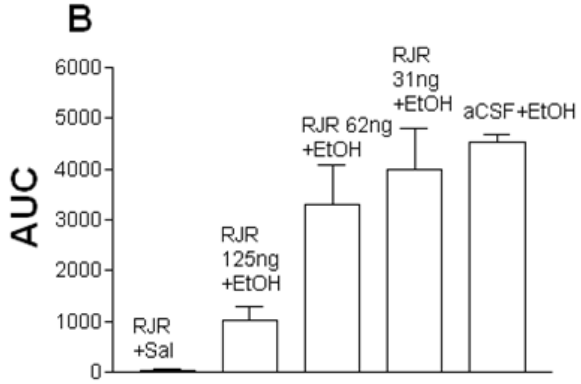
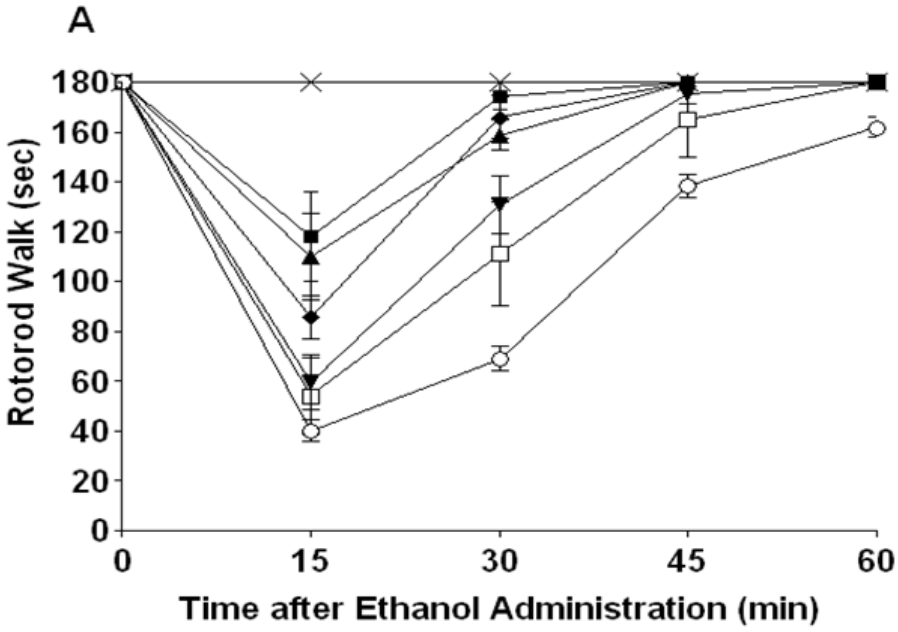
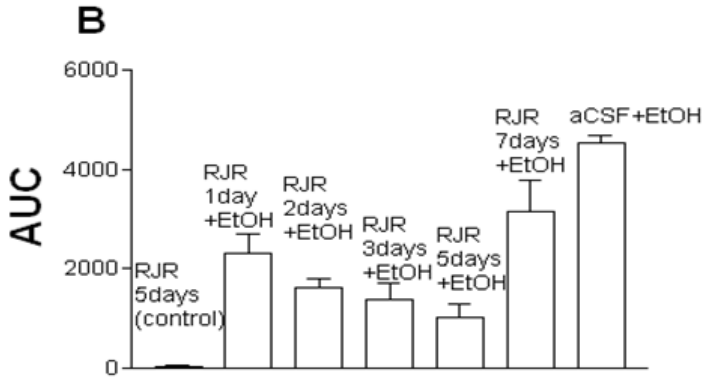

B
AUC
6000
5000
4000
3000
2000
1000
0
RJR
+Sal
RJR
125ng
+EtOH
RJR 62ng
+EtOH
RJR
31ng
+EtOH
aCSF+EtOH

FIGURE 22: The effect of chronic intracerebellar microinfusion (once daily for 1, 2, 3, 5, or 7 days) of RJR-2403 (125ng), 16 hrs following the last intracerebellar RJR microinfusion, on acute ethanol (2g/kg, ip)-induced motor impairment. Each point represents the mean ± S.E.M of 10 mice. **A:** ○ aCSF + Ethanol (2g/kg,i.p.); □ RJR (once daily for 7 days) + Ethanol; ▼ RJR (once daily for 1 days)+Ethanol; ♦RJR (once daily for 2 days) + Ethanol; ▲ RJR (once daily for 3 days) + Ethanol; ■ RJR (once daily for 5 days) + Ethanol; × RJR (once daily for 7 days) + Saline. **B:** The area under the curve (AUC) data are derived from the same treatment presented above in Fig. A; the treatments are shown above the appropriate bars.


A
Rotorod Walk (sec)
180
160
140
120
100
80
60
40
20
0
0
15
30
45
60
Time after Ethanol Administration (min)


B
AUC
6000
4000
2000
0
RJR 5days (control)
RJR 1day +EtOH
RJR 2days +EtOH
RJR 3days +EtOH
RJR 5days +EtOH
RJR 7days +EtOH
aCSF+EtOH

reduction at 15 min in the degree of ethanol ataxia and complete return to normal motor coordination by 30 min post-ethanol (Fig. 23A: ■---■ vs. *---*). When ethanol was administered at 36 or 48 h after the last RJR microinfusion, there was still a pronounced decrease (66%; $p<0.05$) in ethanol ataxia at 30 min post-ethanol (Fig. 23A: ●---●; ▲---▲; *---*). A significant drug treatment and time interaction was observed ($F_{12,156}$ 114 = 2.99; $p<0.001$). Figure 23B presents the relationship between AUC and the time lapsed after RJR-2403 microinfusion when the cross-tolerance was still observable: the longer the time lapsed after RJR-2403 infusion, the larger the AUC and vice versa.

Effect of Repeated Intracerebellar DHβE on Repeated RJR-2403-induced Attenuation of Ethanol Ataxia. To confirm the role of $\alpha_4\beta_2$ subtype in RJR-2403-induced attenuation of ethanol ataxia (Fig. 24), mice were pretreated with DHβE, a selective $\alpha_4\beta_2$ nAChR subtype antagonist for 5 days (once a daily) followed 10 min by RJR-2403. Sixteen hours after the last microinfusion, ethanol ataxia was evaluated. It is clear from Fig. 24 that DHβE significantly reduced RJR-2403's response: 75% at 15 min, 66% at 30 min and 60 % at 45 min with $p<0.05$. Repeated DHβE + RJR-24-3 did not change normal motor coordination when saline was injected instead of ethanol. In Fig. 24B, the same treatment groups as shown in Fig. 24A are expressed as AUC. The various treatments used are shown over the appropriate bar graphs.

FIGURE 23: The effect of repeated intracerebellar (ICB) microinfusion (once daily for 5days) of RJR-2403 (125ng), 16, 36, 48 or 72 hrs following the last ICB RJR microinfusion, on acute ethanol (2g/kg, ip)-induced ataxia. Each point represents the mean ± S.E.M of 10 mice. **A:** ○ aCSF + Ethanol (2g/kg,i.p.); * RJR (72hrs) + Ethanol; ▲ RJR (48hrs) + Ethanol; ● RJR (36hrs) + Ethanol; ■ RJR (16hrs) + Ethanol. **B:** The area under the curve (AUC) data are derived from the same treatments presented above in **A;** the treatments are shown above the appropriate bars.

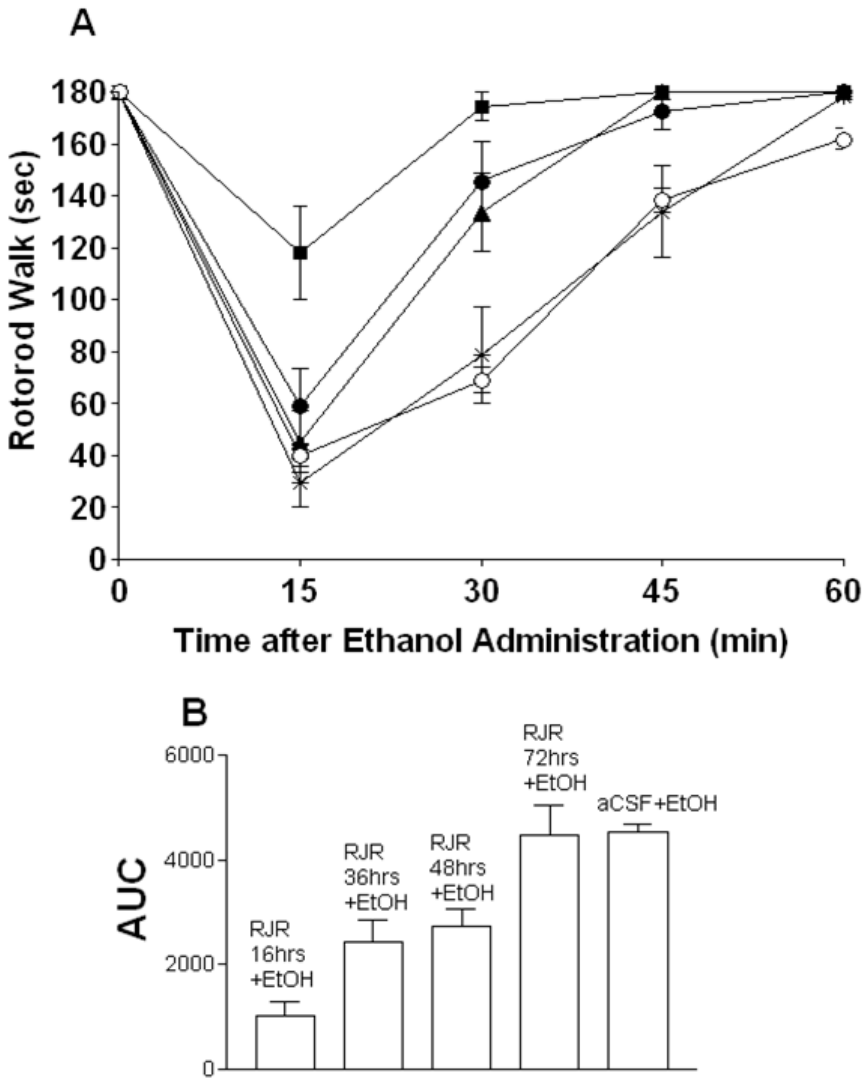
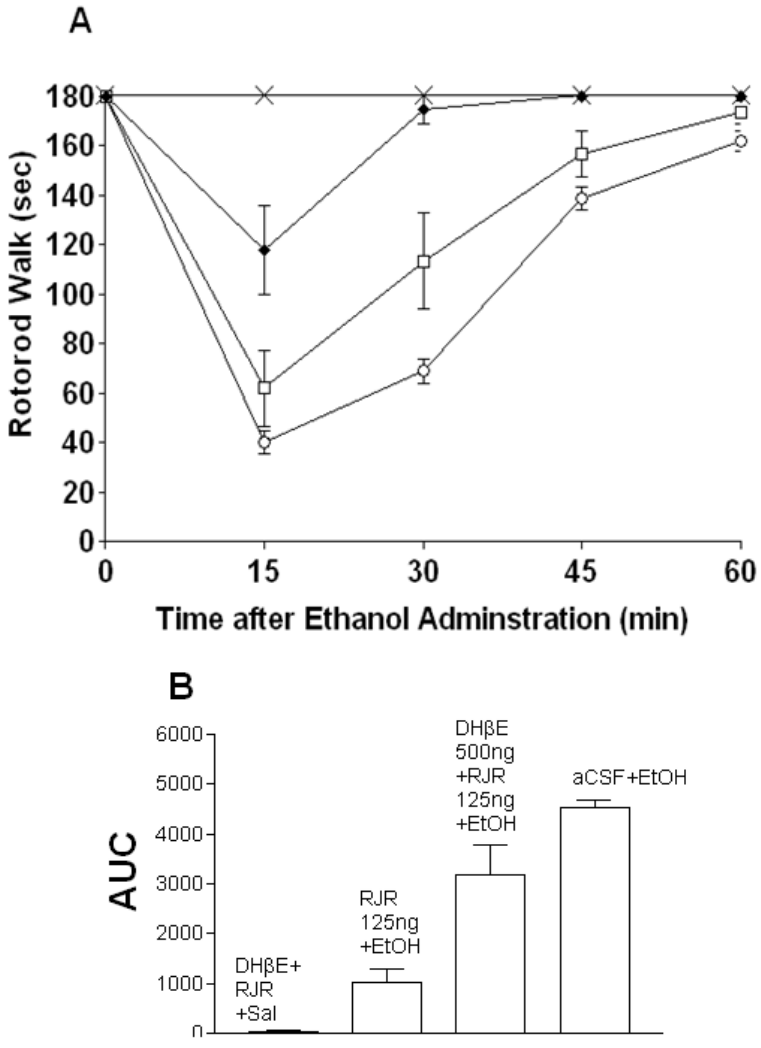

A
Rotorod Walk (sec)
180
160
140
120
100
80
60
40
20
0
0
15
30
45
60
Time after Ethanol Administration (min)
B
AUC
6000
4000
2000
0
RJR
16hrs
+EtOH
RJR
36hrs
+EtOH
RJR
48hrs
+EtOH
RJR
72hrs
+EtOH
aCSF+EtOH

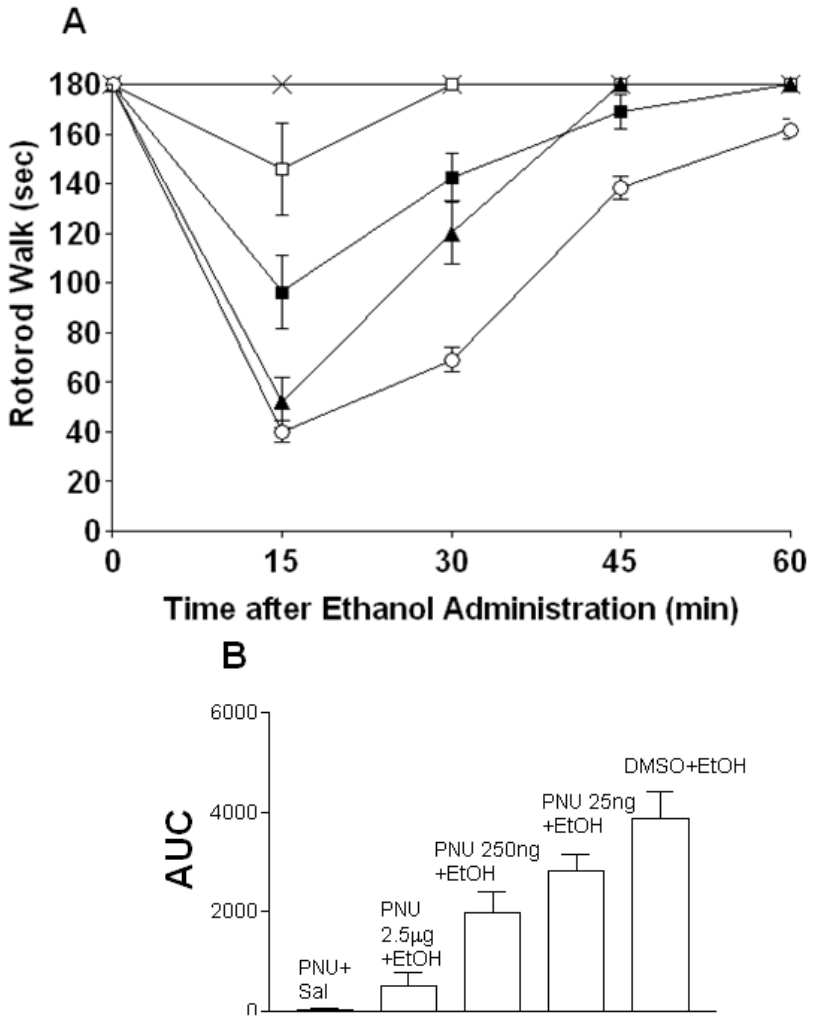
FIGURE 24: The effect of repeated intracerebellar (ICB) microinfusion (once daily for 5 days) of DHβE (500 ng) followed 10 min later by RJR-2403 (125ng) on acute ethanol (2g/kg; i.p.)-induced ataxia. **A:** ○ aCSF + Ethanol (2g/kg,i.p.); □ DHβE + RJR + Ethanol; ♦ RJR +Ethanol; × DHβE + RJR + Saline. Each point represents the mean ± S.E.M of 10 mice. **B:** The area under the curve (AUC) data are derived from the same treatment presented above in **A;** the treatments are shown above the appropriate bars.


A
Rotorod Walk (sec)
180
160
140
120
100
80
60
40
20
0
0
15
30
45
60
Time after Ethanol Adminstration (min)
B
AUC
6000
5000
4000
3000
2000
1000
0
DHβE+
RJR
+Sal
RJR
125ng
+EtOH
DHβE
500ng
+RJR
125ng
+EtOH
aCSF+EtOH

Repeated Intracerebellar microinfusion of PNU 282987 and Ethanol Ataxia: Development of Cross-Tolerance

Dose-Response Relationship between Chronic Intracerebellar PNU-282987 and Acute Ethanol Ataxia. Shown in Fig. 25A is the dose response relationship between repeated ICB PNU-282987 (25ng, 250ng, 2.5µg) treatment and ethanol ataxia that was initiated 16 h after the last PNU-282987 microinfusion. When given by ICB microinfusion for 3 consecutive days (once daily), PNU-282987 dose dependently diminished acute ethanol induced ataxia when the animals were evaluated 16 h after the last treatment with PNU-282987. This indicated the development of cross-tolerance between PNU-282987 and ethanol ataxia. The PNU-282987 dose of 2.5µg caused the maximum reduction (73%; $p<0.05$) of ethanol ataxia at 15 min which was an indication of the highest degree of cross-tolerance development followed by that produced by PNU-282987 250ng dose (41%; $p<0.05$). The animals in the 2.5µg and 250 ng treatment groups regained their normal motor coordination by 30 - 45 min post-ethanol administration, respectively. The smallest 25ng dose did result in a significant reduction in ethanol ataxia at 30 (42%; $p<0.05$), 45 (100%), and 60 min (100%) post-ethanol administration. A significant drug treatment and time interaction was observed ($F_{9,102}=7.17$; $p<0.001$). Intracerebellar pretreatment with the α_7-selective antagonist, methyllycaconitine (6 ng; once daily for 3 days) 10 min prior to ICB microinfusion of PNU-282987 nearly abolished the ability of PNU-282987 to induce the development of cross-tolerance to ethanol ataxia. The methyllycaconitine + PNU-282987 + saline control treatment group did not alter normal motor coordination. Figure 25B shows the expression of the data presented in Fig. 25A as the AUC. The AUC is directly related to

FIGURE 25: The effect of repeated intracerebellar (ICB) microinfusion of PNU-282987 (25ng, 250ng or 2.5µg; once daily for 3days), 16 hrs following the last ICB PNU-282987 microinfusion, on acute ethanol (2g/kg, ip)-induced ataxia. Each point represents the mean ± S.E.M of 10 mice. **A:** ○ aCSF + Ethanol (2g/kg,i.p.); ▲ PNU 25ng + Ethanol; ■ PNU 250ng + Ethanol; □ PNU 2.5µg + Ethanol; × PNU 2.5µg + Saline. **B:** The area under the curve (AUC) data are derived from the same treatments presented above in A; the treatments are shown above the appropriate bars.


A
Rotorod Walk (sec)
180
160
140
120
100
80
60
40
20
0
0
15
30
45
60
Time after Ethanol Administration (min)
B
AUC
6000
4000
2000
0
PNU+
Sal
PNU
2.5µg
+EtOH
PNU 250ng
+EtOH
PNU 25ng
+EtOH
DMSO+EtOH

degree of ataxia i.e., the greater the degree of ataxia the greater will be the AUC associated with decreasing dose of PNU-282987.

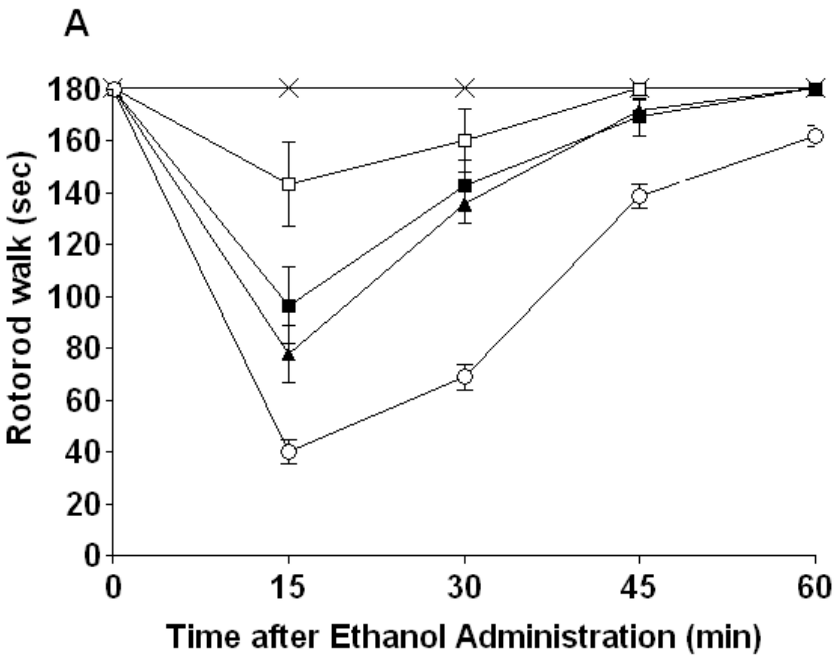
Initiation Time for Development of Cross-Tolerance Between Intracerebellar PNU-282987 and Acute Ethanol Ataxia. Figure 26A illustrates the time for onset of cross tolerance between repeated ICB PNU-282987 microinfusion and acute ethanol ataxia. The mice were evaluated 16 h after last microinfusion of PNU (250ng). This dose of PNU-282987 was selected based on our acute studies in which both 250 ng and 2.5µg doses of PNU-282987 produced nearly the same maximal degree of attenuation of ethanol ataxia. In the present study the dose-response data (Fig. 25A) indicates that the 250 ng dose of PNU-282987 is of intermediate potency in attenuating ethanol ataxia. A single microinfusion of PNU-282987 (i.e. 1-day treatment group) caused the greatest reduction in ethanol ataxia indicating the onset of cross-tolerance between PNU-282987 and acute ethanol ataxia (Fig. 26A □---□). However, when the same dose (250 ng) of PNU-282987 was repeated (once daily) for 3 and 5 days, the degree of cross-tolerance as measured by reduction in ethanol ataxia, started to taper off. There was virtually no difference in the degree of cross-tolerance between 3 and 5 days of PNU-282987 treatment as both caused a 42% and 34% decrease in ataxia at 15 min and about 65% at 30 min with $p<0.05$, respectively. It is clear from the Fig. 26A that the cross-tolerance was maximal after 1 day (single microinfusion) of PNU-282987 treatment. A significant drug treatment and time interaction was observed ($F_{9, 115} = 3.09$; $p<0.001$). There was no effect of PNU-282987 when a 250 ng dose was microinfused for 3 days followed 16 h after the last PNU-282987 treatment with saline injection instead of ethanol (Fig. 26A

----). In Fig. 26B, the same treatment groups as shown in Fig. 26A are expressed as AUC. The various treatments used are shown over the appropriate bar graphs.

Duration of Cross-Tolerance between Intracerebellar PNU-282987 and Acute Ethanol Ataxia. Figure 27A shows the time course of the development of cross-tolerance between PNU-282987 and acute ethanol ataxia. The ataxic response of an acute ethanol test dose was evaluated at 16, 36, 48, 96 h after a one time only ICB microinjection of 250ng dose of PNU-282987. PNU-282987 produced marked attenuation of ethanol ataxia at 16, 36 and 48 h following its microinfusion versus the aCSF + Ethanol controls. The animals in these three groups regained 100% of their normal motor coordination within 30 min post-ethanol administration (Fig. 27A: ∆---∆; ▲---▲; *---*). At 96 h post- PNU-282987 microinfusion, the animals were totally free of any attenuating effect of PNU-282987 on ethanol ataxia. The Rotorod response curve of animals in the 96 h post- PNU-282987 group overlapped the ethanol control (Fig. 27A; □---□ vs. ○---○) response curves. Overall, a significant drug treatment and time interaction was observed ($F_{12, 135} = 15.5$; $p < 0.001$). Pretreatment with hexamethonium (1 µg; ICB) produced a complete blockade in PNU's effect when evaluated 48 h after PNU-282987 microinfusion. As shown in Fig. 27B, AUC is largest for aCSF + ethanol and 96 h post PNU-282987 injection there was no attenuation of ethanol ataxia. The AUC decreased correspondingly as the time interval between PNU-282987 microinfusion and acute ethanol ataxia becomes reduced i.e., from 48h to 36 h to 16h.

Effect of Repeated Intracerebellar Methyllycaconitine on Repeated PNU-282987-induced Attenuation of Ethanol Ataxia. Figure 28 depicts the effect of pretreatment of methyllycaconitine (6 ng), an α_7-subtype selective antagonist on PNU-282987-induced

FIGURE 26: The effect of repeated intracerebellar (ICB) microinfusion of PNU-282987 (250ng; once daily for 1, 3 or 5 days), 16 hrs following the last ICB PNU microinfusion, on acute ethanol (2g/kg, ip)-induced ataxia. Each point represents the mean ± S.E.M of 10 mice. **A:** ○ aCSF + Ethanol (2g/kg,i.p.); ▲ PNU (5 days) + Ethanol; ■ PNU (3 days)+Ethanol; □ PNU (1days) + Ethanol; × PNU (3D) + Saline. **B:** The area under the curve (AUC) data are derived from the same treatments presented above in **A;** the treatments are shown above the appropriate bars.


A
Rotorod walk (sec)
180
160
140
120
100
80
60
40
20
0
0
15
30
45
60
Time after Ethanol Administration (min)

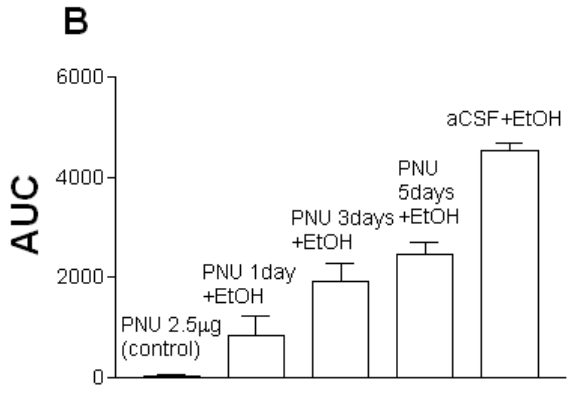
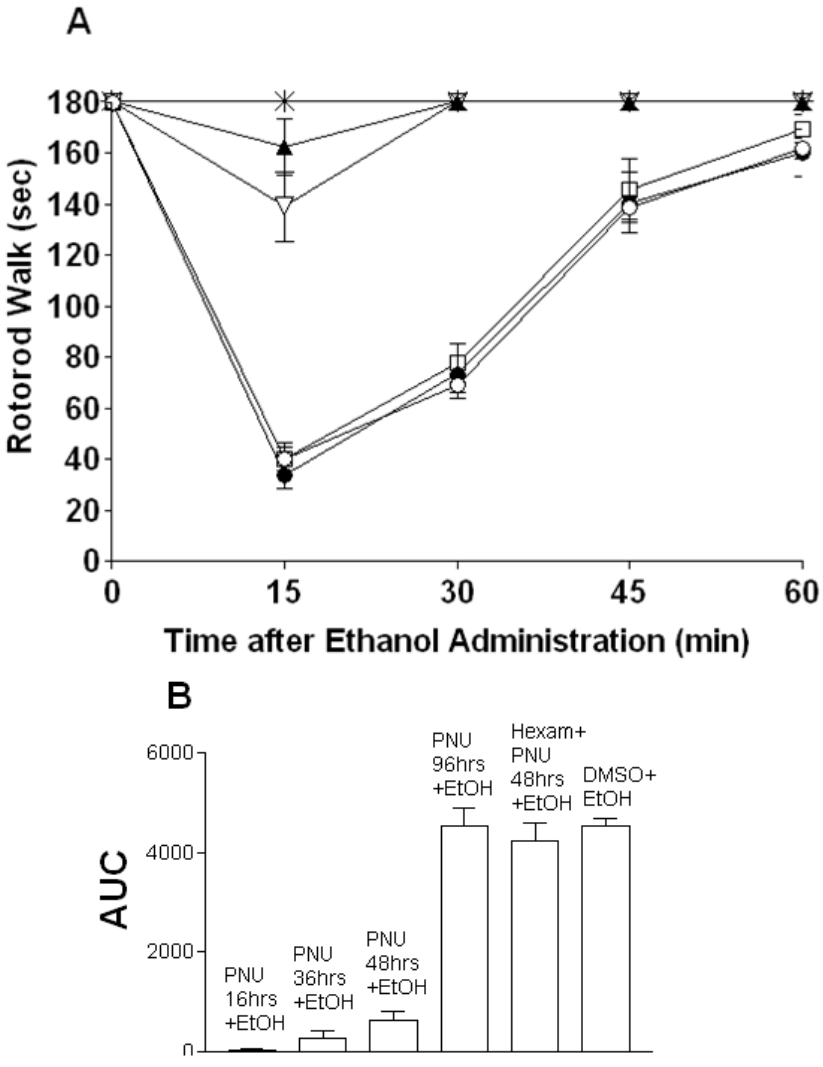

B
AUC
6000
4000
2000
0
PNU 2.5μg
(control)
PNU 1day
+EtOH
PNU 3days
+EtOH
PNU
5days
+EtOH
aCSF+EtOH

FIGURE 27: The effect of a single intracerebellar (ICB) microinfusion of PNU-282987 (250 ng), 16, 36, 48 or 96 hrs following the last ICB microinfusion off PNU, on acute ethanol (2g/kg, ip)-induced ataxia. **A:** ○ aCSF + Ethanol (2g/kg,i.p.); ● Hexamethonium + PNU (16hr)+Ethanol; □ PNU (96hrs) + Ethanol; ∆ PNU (48hrs) + Ethanol ; ▲ PNU (36hrs) + Ethanol; * PNU (16hrs) + Ethanol. Each point represents the mean ± S.E.M of 10 mice. **B:** The area under the curve (AUC) data are derived from the same treatment presented above in **A;** the treatments are shown above the appropriate bars.

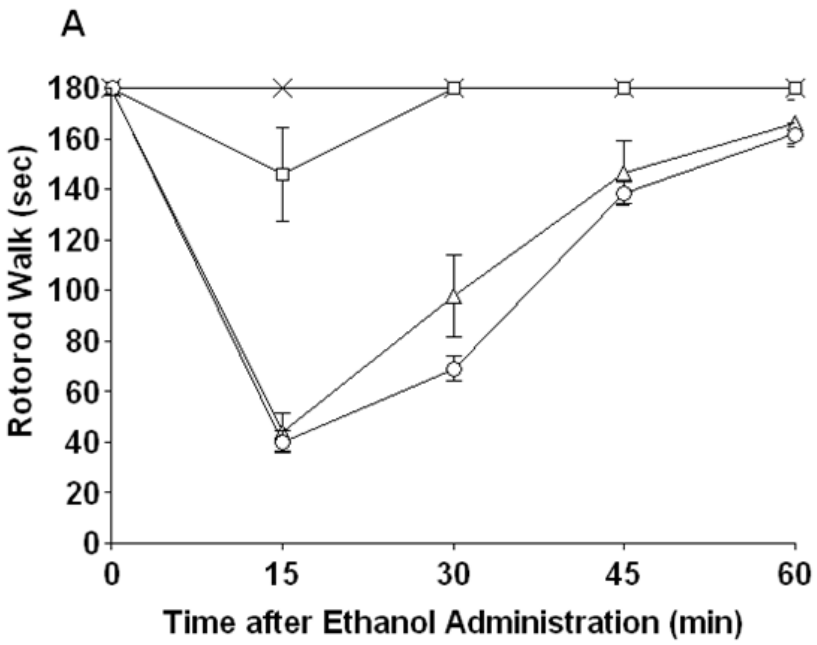

A
Rotorod Walk (sec)
180
160
140
120
100
80
60
40
20
0
0
15
30
45
60
Time after Ethanol Administration (min)
B
AUC
6000
4000
2000
0
PNU 16hrs +EtOH
PNU 36hrs +EtOH
PNU 48hrs +EtOH
PNU 96hrs +EtOH
Hexam+ PNU 48hrs +EtOH
DMSO+ EtOH

attenuation of ethanol ataxia. This experiment was performed to confirm the involvement of the α_7 nAChR subtype in the mediation of the action of PNU-282987 against ethanol ataxia (Fig. 28) Mice were repeatedly treated with methyllycaconitine for 3 days (once per day) followed 10 min later by PNU-282987 and ethanol ataxia was tested 16 h after the third microinfusion. As shown in Fig. 28, methyllycaconitine completely abolished the PNU-282987 -induced attenuation of ethanol ataxia. However, the combination of methyllycaconitine + PNU-282987 did not alter normal motor coordination (Fig. 28; x---x). In Fig. 28B, the same treatment groups as shown in Fig. 28A are expressed as AUC. The various treatments used are shown over the appropriate bar graphs.

Effect of Acute Intracerebellar RJR-2403/ PNU-282987 in the Absence or Presence of Systemic Ethanol on Cerebellar Nitric Oxide Levels

Figure 29 shows the changes in the cerebellar cortical in NO_x levels due to acute ethanol treatment versus saline controls in mouse cerebellar tissue homogenates (Panel A). Acute ethanol i.p. significantly reduced the total NO_x levels by 44% ($p<0.05$). Panel B illustrates the effect of microinfusion of acute ICB RJR-2403, PNU-282987 each alone and in combination with acute ethanol. It is clearly shown that both agonists (RJR-2403, PNU-282987) resulted in dramatic augmentation of NO_x versus contralateral controls: 750% ($p<0.001$) for RJR-2403 and 600% ($P<0.01$) for PNU-282987 as determined by DAN colorimetric method. Concurrent administration of ICB RJR-2403 or PNU-282987 in the presence of ethanol did enhance NO_x compared with contralateral controls but the values were not significant different from the respective agonist alone. Overall a significant difference between drug treatment and controls was observed ($F_{5,39}=9.41$, $p<0.000$).

FIGURE 28: The effect of repeated intracerebellar (ICB) microinfusion (once daily for 3 days) of methyllycaconitine (6 ng) followed 10 min later by PNU-282987 (2.5µg) on acute ethanol (2g/kg; i.p.)-induced ataxia. **A:** ○ aCSF + Ethanol (2g/kg,i.p.); ∆ Methyllycaconitine + PNU + Ethanol; □ PNU + Ethanol; × Methyllycaconitine + PNU + Saline. Each point represents the mean ± S.E.M of 10 mice. **B:** The area under the curve (AUC) data are derived from the same treatment presented above in **A;** the treatments are shown above the appropriate bars.


A
Rotorod Walk (sec)
180
160
140
120
100
80
60
40
20
0
0
15
30
45
60
Time after Ethanol Administration (min)

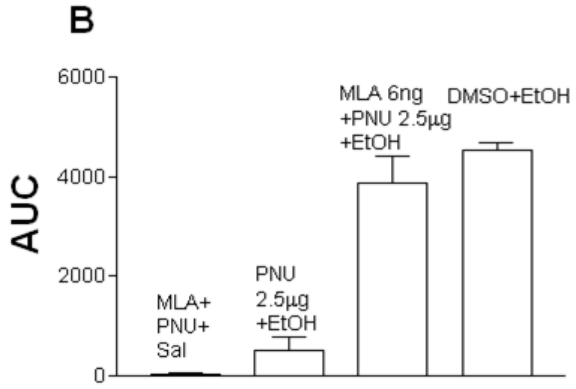
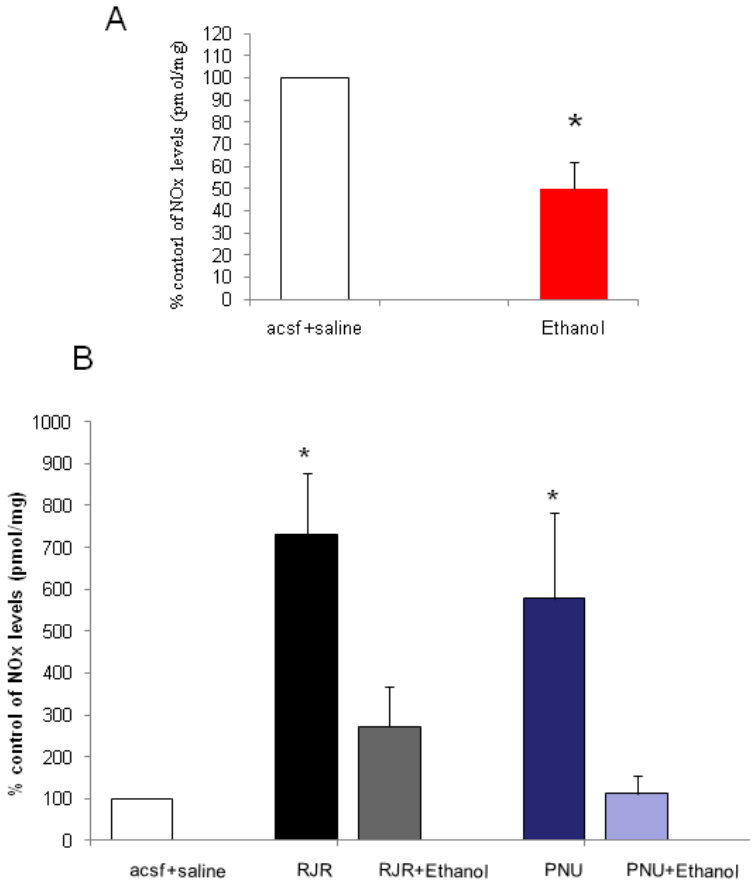

B
AUC
6000
4000
2000
0
MLA+
PNU+
Sal
PNU
2.5µg
+EtOH
MLA 6ng
+PNU 2.5µg
+EtOH
DMSO+EtOH

FIGURE 29. A: The effect of acute ethanol versus saline challenge on NOx levels on the mouse cerebellar homogenates. **B:** Changes in NOx levels following acute RJR-2403 (125ng) or PNU-282987 (250ng) alone and in combination with ethanol. Each point represents the mean ± S.E.M of 10 mice. *$p<0.05$ vs. controls.


A
% control of NOx levels (pmol/mg)
120
110
100
90
80
70
60
50
40
30
20
10
0
*
acsf+saline
Ethanol
B
% control of NOx levels (pmol/mg)
1000
900
800
700
600
500
400
300
200
100
0
*
*
acsf+saline
RJR
RJR+Ethanol
PNU
PNU+Ethanol

Effect of Repeated Intracerebellar RJR-2403/ PNU-282987 in the Absence or Presence of Ethanol on Cerebellar Nitric Oxide Levels

Figure 30 shows the effect of acute ethanol (Panel A) and of repeated intracerebellar RJR-2403 and PNU-282987 alone or in the presence of ethanol (Fig. 30B) on NO_x (total nitrate + nitrite) levels in mouse cerebellar tissue homogenates. Ethanol (2g/kg;i.p.) administration resulted in significant reduction (42%) of NO_x (Panel A). On the other hand, there was a dramatic increase in NO_x levels following RJR-2403 and PNU-282987 microinfusions. The cerebellar NO_x levels determined flourometrically were markedly enhanced (500%) at 16h following ICB RJR-2403 (125ng) and about 800% after PNU-282987 (250ng) microinfusions (Panel B). Intracerebellar pretreatment with RJR-2403 or PNU-282987 completely prevented the reduction in the cerebellar NO_x due to ethanol. The basal concentration of NO_x was measured following ICB microinfusion of aCSF.

Immunohistochemical Localization of α_4 and α_7 Subtypes in Mouse Cerebellum

Figure 31 depicts the localization of the two subtypes of nAChRs, α_4 and α_7, within the cerebellar cortex. Immunoreactivity for both the α_4 and α_7 nAChR subtypes in the molecular and Purkinje cell layers of the cerebellar cortex was moderate. The expression pattern of both subtypes appeared identical at these sites with α_4 levels being slightly denser than those of α_7**.** The immunochemical data demonstrated relatively low α_4 and α_7 staining in granule layer (Fig. 31).

FIGURE 30. A: The effect of acute ethanol or saline challenge on NOx levels versus aCSF/or contra-lateral controls on mouse cerebellar homogenates. **B:** The changes in NOx levels following 16 h of repeated RJR-2403 (125ng; ICB, once daily for 5 days) or PNU (250ng; ICB, once daily for 3 days) alone and in combination with ethanol are shown. Each point represents the mean ± S.E.M of 10 mice. *$p<0.05$ vs. controls.

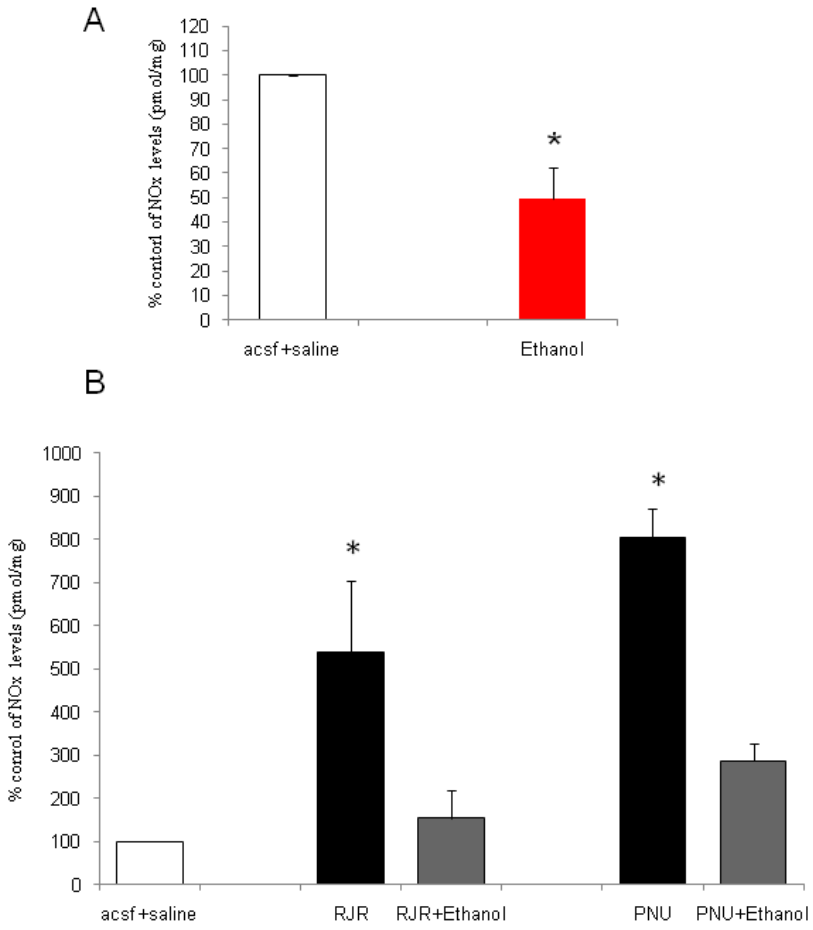
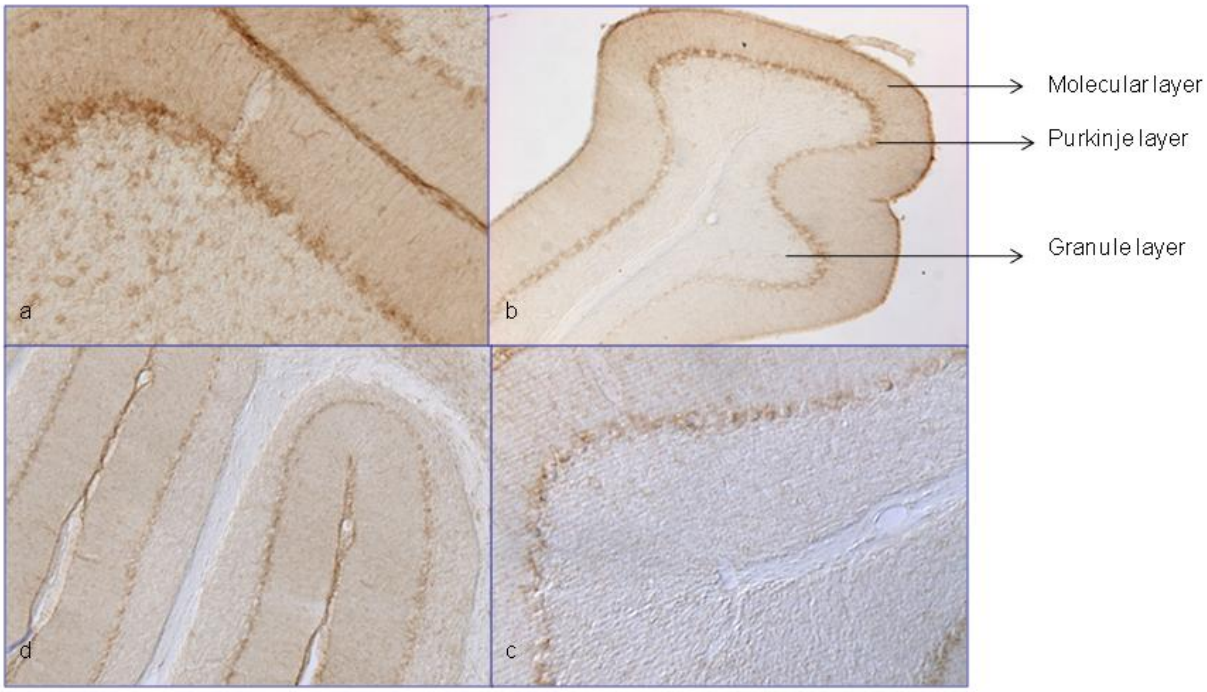

A
% control of NOx levels (pmol/mg)
120
110
100
90
80
70
60
50
40
30
20
10
0
*
acsf+saline
Ethanol
B
% control of NOx levels (pmol/mg)
1000
900
800
700
600
500
400
300
200
100
0
*
*
acsf+saline
RJR
RJR+Ethanol
PNU
PNU+Ethanol

FIGURE 31: Immunohistochemcial localization of α_4 (a,b)and α_7 (c,d) subtypes in mouse cerebellum.


a
b
c
d
Molecular layer
Purkinje layer
Granule layer

CHAPTER FOUR: DISCUSSION

It is of grave concern to public health authorities that two highly addictive psychoactive drugs (i.e. nicotine and ethanol) are widely abused together in many societies (Romberger and Grant, 2004; Room, 2004). This tendency toward co-abuse leads to higher mortality and morbidity rates in co-abusers than that observed for either drug alone. This fact demands a thorough understanding of the mechanisms implicated in co-addiction and requires investigating the inter-active relationship between the two substances of abuse.

Earlier work in this laboratory revealed that ICB microinfusion of nicotine markedly and dose-dependently attenuated ethanol ataxia. The functional interaction between the two psychoactive substances was noted when nicotine was administered acutely (Dar et al., 1993, 1994; Al-Rejaie and Dar, 2006a) as well as repeatedly (Al-Rejaie and Dar, 2006b). We also report the participation of the cerebellar NO system in mediating or antagonizing ethanol ataxia. This dissertation research, in extension of an earlier work, investigates the participation of two predominant nAChR sutbypes $\alpha_4\beta_2$ and α_7 in mediating of nicotine's attenuation of ethanol ataxia. Achievement of this goal was accomplished by modulating the nAChR subtype $\alpha_4\beta_2$ and α_7 function using selective subtype agonists and antagonists in acute as well as repeated treatment paradigms. The correlation of cerebellar NOx levels with ethanol ataxia and its attenuation was also established in this project.

Behavioral Interaction Between Acute $\alpha_4\beta_2$ and α_7 Selective Drugs and Ethanol Ataxia

Role of $\alpha_4\beta_2$ subtypes. We have previously demonstrated that a marked attenuation of ethanol ataxia occurred following ICB microinfusion of nicotine which suggested a functional interaction between nicotine and ethanol (Al-Rejaie and Dar, 2006a,b,c; Dar et al., 1993, 1994). The functional interaction between these two psychoactive drugs was observed following acute and chronic ICB microinfusion of nicotine (Al-Rejaie and Dar, 2006b,c).

The first aim of the study was to delineate the role of cerebellar $\alpha_4\beta_2$ subtype (the most abundant subtype of nAChR system) in nicotine-induced modulation of ethanol ataxia. The nAChR $\alpha_4\beta_2$-selective agonist, RJR-2403, like nicotine, markedly attenuated motor impairment due to ethanol in a dose-dependent manner which indicated a role of $\alpha_4\beta_2$ subtype in nicotine-ethanol functional interaction. The dose-dependency of RJR-2403's action presumably is reflective of its selective action at the $\alpha_4\beta_2$ subtype. When given alone, RJR-2403 neither altered the normal motor coordination nor produced any overt behavioral hyperactivity. Further evidence for the involvement of $\alpha_4\beta_2$ subtype in the functional interactions between nicotine and ethanol was provided by the results of experiments in which DHβE was used as an ICB pretreatment. Pretreatment with DHβE, a selective $\alpha_4\beta_2$ antagonist, blocked the attenuation by RJR-2403 of ethanol ataxia. This indicates that attenuation of ethanol ataxia is an $\alpha_4\beta_2$ nAChR subtype-mediated phenomenon and that the $\alpha_4\beta_2$ subtype is an important participating factor in the functional interaction between nicotine and ethanol. The ability of DHβE to block the attenuating effect of the RJR-2403 in a dose-dependent fashion strongly suggests an involvement of

$\alpha_4\beta_2$ subtype in nicotine-ethanol interaction. Nevertheless, DHβE was unable to prevent the attenuating action of RJR-2403 when the order of administration of both drugs was reversed (i.e., RJR-2403 is administered prior to DHβE microinfusion). This result is most likely because DHβE is a competitive $\alpha_4\beta_2$ antagonist (Damaj et al., 1995b) and may compete with RJR-2403 for the same binding sites. Therefore, whichever drug is administered first occupies the binding sites and prevents the binding of other drug. This phenomenon may be consistent with the observed dose-dependency of DHβE in the attenuation of RJR-2403 action.

The hypothesis that $\alpha_4\beta_2$ subtype plays an important role in the mediation of the functional interaction between the two psychoactive drugs was further supported by the results of DHβE + nicotine Rotorod experiments. The ICB microinfusion of nicotine markedly attenuated ethanol ataxia, with the highest (5 ng) dose virtually abolishing ethanol ataxia (Fig. 10). Interestingly, however, following ICB pretreatment with DHβE, interestingly we observed a dose-related marked antagonism of nicotine-induced attenuation of ethanol ataxia. This observation supports the view that nicotine-induced attenuation of ethanol ataxia was, in fact, mediated through nAChR of the $\alpha_4\beta_2$ subtype. Since the highest dose of DHβE completely abolished the attenuating effect of nicotine on ethanol-induced ataxia, it may also suggest a primary role of $\alpha_4\beta_2$ subtype in the functional interaction between nicotine and ethanol. The response curve of DHβE (750 ng) + nicotine in the presence of ethanol was nearly identical to that of aCSF + ethanol group (Fig. 10).

In a previously reported study (Al-Rejaie and Dar, 2006a,b,c), the attenuating effect of ICB nicotine on ethanol-indcued ataxia was observed only when nicotine was

microinfused first, followed by ethanol administration, and not vice versa. However, in the present investigation RJR-2403 was found to be equally effective in attenuating the ethanol-induced ataxia regardless of the order of its microinfusion and ethanol injection. There was no difference in the response curves of RJR-2403 + ethanol and ethanol + RJR-2304. Thus, in the case of nicotine the initial activation of nAChRs tantamount to a "priming effect" was essential to counter ethanol ataxia because nicotine was ineffective when administered post-ethanol. The previous observation (Al-Rejaie and Dar, 2006a) that nicotine must be administered first in order to observe its attenuating effect on ethanol ataxia and that it was totally ineffective when given post-ethanol administration may suggest that nicotine perhaps exerts a "priming" effect on the downstream signaling pathways that eventually results in elimination of the ataxic effect of ethanol. On the other hand, RJR-2403 did reverse ethanol ataxia whether injected pre- or post-ethanol. At present, we do not have an explanation for the observed difference between nicotine and RJR-2403. It is plausible that by giving ethanol first instead of nicotine; ethanol produces well known membrane fluidization. The latter membrane effect can alter the membrane functions and may change the microenvironment to the extent that nicotine by virtue of its different chemical structure and perhaps pharmacokinetic properties from RJR-2403 was unable to activate its receptors.

Finally, additional evidence in support of the role of $\alpha_4\beta_2$ subtype in the functional interaction between nicotine and ethanol was provided by the results of the Rotorod experiments using antisense and missense oligonucleotides against $\alpha_4\beta_2$ subtype of nAChR. Nicotinic acetylcholine receptor subtype $\alpha_4\beta_2$ antisense oligonucleotide hybridizes with target mRNA and results in protein-specific translation arrest (Bitner et al., 2000).

This action was the basis to obtain direct evidence in further support of our hypothesis that $\alpha_4\beta_2$ subtype primarily mediates the functional interaction between nicotine and ethanol. We relied on an already published phosphorothioate-modified oligonucleotides corresponding to the initial 5′ nucleotide residues (2-21) of the rat α_4 subunit (Bitner et al., 2000). After antisense treatment, RJR-2403 was unable to attenuate ethanol ataxia. This observation served the dual purpose to: (i) indicate the effectiveness of antisense; and: (ii) confirm that the nAChR $\alpha_4\beta_2$ subtype mediates the nicotine-induced attenuation of ethanol ataxia. The effectiveness of antisense was also confirmed by the observation of a significant decrease in α_4 receptor protein expression based on Western blot data vs. missense treatment (Fig. 12). Therefore, the Rotorod data with RJR-2403 demonstrating an involvement of the $\alpha_4\beta_2$ subtype in the nicotine – ethanol functional interaction was in agreement with the antisense oligonucleotide/Western blot data confirming the important participation of nAChR subtype $\alpha_4\beta_2$ in the nicotine and ethanol functional interaction.

The antisense oligonucleotide-treated mice recovered from ethanol-induced ataxia relatively sooner following microinfusion of RJR-2403 than the control aCSF + ethanol treated mice (with no oligonucleotide pretreatment). This result means that the antisense treated mice still retained some expression of the α_4 subtype and further supported by our immunoblot experiments where a faint but much reduced signal of the α_4 protein was detected after antisense treatment. Despite the fact that we used a dose 10 times higher than that reported by Bitner et al, (2000), we were unable to observe any visual or histological signs of toxicity. This is probably because they infused drugs i.c.v. from where drugs could have permeated the whole brain while we infused drugs/oligonucleotides directly into the cerebellar cortex to obtain a localized action. We have previously

demonstrated that the diffusion of drug molecules following ICB microinfusion remains confined within a 2 mm area from the site of microinfusion (Meng and Dar, 1996). The ICB administration of RJR-2403 produced a dose-dependent attenuation of ethanol ataxia which exhibited a pharmacological profile nearly identical to that of nicotine. Briefly, this part of the project demonstrated that nicotine-induced attenuation of ethanol ataxia is primarily mediated by $\alpha_4\beta_2$ subtype.

Role of α_7 subtype. After establishing the role of $\alpha_4\beta_2$ nAChR subtype in nicotine-induced reduction of ethanol ataxia, we sought to delineate the role of the α_7 nAChR subtype in nicotine-ethanol's functional interaction. And for that we chose PNU-282987, a potent and selective α_7 nAChR subtype agonist with a K_i value of 27 nM. We observed that ICB microinfusion of the α_7-selective subtype agonist, PNU-282987, markedly and dose-dependently antagonized ethanol ataxia. The dose-dependency of the attenuating action of PNU-282987 on ethanol ataxia is strongly supportive of a role for α_7 subtype nAChR in the established observation of nicotine-ethanol functional interaction. PNU-282987, similar to nicotine, significantly attenuated motor impairment due to ethanol administration. Like nicotine, ICB microinfusion of PNU-282987 alone when followed by saline instead of ethanol injection failed to alter normal motor coordination or produced little overt behavioral hyperactivity.

Further evidence for the role of α_7 subtype nAChR and the selective action of PNU-282987 was provided by the results of the experiments in which pretreatment with α_7-subtype selective antagonist methyllycaconitine, obliterated PNU-282987-induced attenuation of ethanol ataxia. However, pretreatment with methyllycaconitine, an α_7 subtype-selective antagonist, did not alter the action of the $\alpha_4\beta_2$ subtype selective agonist,

RJR-240 in attenuating ethanol ataxia. The failure of the ICB dose (6 ng; Fig. 16**A ▼---▼)** of methyllycaconitine to block the attenuating effect of RJR-2403 on ethanol ataxia demonstrated the relative selectivity of methyllycaconitine as an antagonist of nAChR α_7–subtype at this dose. The results also suggested that at the dose range selected in the present study, both methyllycaconitine and PNU-282987 primarily acted at α_7 subtype containing nAChR (Fig. 16**A**).

We also observed that ICB pretreatment of methyllycaconitine significantly antagonized nicotine-induced attenuation of ethanol ataxia in a dose-related manner suggesting, that at least in part, nicotine-induced attenuation of ethanol ataxia was indeed caused through participation of nAChR α_7 subtype as well. The highest (12 ng) dose of methyllycaconitine virtually abolished the attenuating effect of nicotine indicating the significance of the role of α_7 subtype nAChR. However, unlike nicotine which required an initial activation/priming of nAChR because it did not exhibit attenuation of ethanol ataxia when microinfused after the administration of ethanol (Al-Rejaie and Dar, 2006a,b,c), PNU-282987 exhibited an ability to antagonize the ethanol ataxia irrespective of whether ethanol is injected prior to or following ICB microinfusion of PNU-282987 (data not shown). This was also true with respect to RJR-2403 which demonstrated antagonism to ataxia whether if ethanol was administered before or after ICB microinfusion of RJR-2403 (Taslim et al., 2008). It is difficult to provide any plausible explanation for the difference between nicotine-ethanol and RJR-2403- or PNU-282987-ethanol interaction. It is probable that some pharmacokinetic or structural differences might have accounted for such variation in their pharmacological action. It is also proposed that ethanol is known to alter membrane

fluidity and thereby may alter nAChR target sites inaccessible to nicotine but not to RJR-2403 or PNU-282987.

Further evidence was sought in support of the involvement of the α_7 subtype in the attenuating the effect of nicotine in ethanol-induced ataxia by the use of antisense and missense oligonucleotides against α_7 subtype of nicotinic receptor. The antisense sequence was complementary to the translation initiation site of the nAChR α_7 subtype. The oligonucleotide antisense sequence was employed to interrupt the protein synthesis of α_7 protein. The antisense and the missense were administered as chronic ICB treatment in order to observe a possible alteration in the levels of the α_7 subtype proteins. The use of α_7 oligonucleotide was to knock down mRNA transcript and its resultant α_7 protein. Following chronic antisense treatment, PNU-282987 was ineffective in overcoming ethanol ataxia unlike in animals with missense treatment indicating interruption in α_7 subtype synthesis. However, PNU-282987's action returned within 30 min. We were not able to observe a significant difference in the α_7 subtype protein expression in Western blot analyses. It appears that only a slight alteration in α_7 protein caused a significant reduction in the Rotorod behavioral response at 15 and 30 min post-ethanol administration. It can be speculated that the discrepancy between the results of behavioral and immunoblot experiments might be due to nonspecific binding of α_7 nAChR antibodies as reported by Herber et al. (2004). These investigators tested different commercially available antibodies on various brain samples obtained from non-transgenic (α_7^+) and null mutant (α_7^-) mice. They found that α_7 antibodies nonspecifically labeled proteins in both samples and that there were no perceptible differences in binding pattern between samples from α_7^+ and α_7^- mice. This points towards questionable specificity of commercially available α_7 antibodies.

Moreover, it is also possible that oligonucleotides were degraded and therefore could not reduce α_7 protein expression. Unlike α_4 oligonucleotides, α_7 oligonucleotides produced some signs of toxicity (e.g. weight loss, bleeding), which also indicated that these oligonuceotides might have produced some tissue damage, although it was not apparent in our histological sections. If the latter possibility is true then it is possible that the inability of animals to walk after oligonucleotides and PNU-282987 treatment was due to some immune related toxicity in the cerebellar cortex rather than due to reduction in α_7 protein expression. In conclusion, the results of our acute experimental protocol support an important role of $\alpha_4\beta_2$ and α_7 nAChR subtypes in the nicotine-induced attenuation of ethanol ataxia.

Behavioral Interaction Between Repeated $\alpha_4\beta_2$ and α_7 Selective Drugs and Ethanol Ataxia

Since the nature of abuse of nicotine in the form of cigarette smoking and of ethanol as alcoholic beverage is chronic, it was important to investigate the functional consequences of the repeated use of these psychoactive substances. It is the co-abuse of both drugs that poses the greater challenge to public health care providers. Essentially, this constituted the basis of this research project. Our previous work demonstrated the development of cross tolerance between repeated ICB nicotine and ethanol ataxia. Thus, the second aim of this project was to see if $\alpha_4\beta_2$ and or α_7 subtypes of nAChRs are important in the development of cross-tolerance between ICB chronic nicotine and acute ethanol that we previously observed and reported.

Cross-Tolerance between Repeated α4β2 selective agonist, RJR-2403 and Acute Ethanol Ataxia. According to the results of our repeated treatment protocol, tolerance develops to acute ethanol ataxia in mice repeatedly exposed to RJR-2403 by ICB microinfusions. The development of cross tolerance was $\alpha_4\beta_2$ dependent as pretreatment with selective antagonists prevented the subtype agonists-induced tolerance to ethanol ataxia. Cross-tolerance between RJR-2403 and ethanol ataxia was dose- and time-dependent: peaked after 5 days of exposure and lasted until 48 h following the last RJR-2403 microinfusion. The evaluation of ethanol ataxia was performed only 16 h after the last RJR-2403 microinfusion. This time period was considered reasonably adequate during which RJR-2403 was expected to have been either eliminated from the animal system or converted to inactive metabolites. This time frame was especially appropriate for RJR-2403 due to its biological half-life of 2 h. Seven days of repeated ICB microinfusion of RJR-2403 resulted in significantly diminished Rotorod response (i.e., a markedly less attenuation of ethanol ataxia was observed). In fact the attenuation of ethanol ataxia was so small that the response curve appeared comparable to ethanol control response curve. Another interesting observation was that 7 days of RJR-2403 treatment was accompanied by abolishment of RJR-2403-induced attenuating effects on ethanol ataxia. The explanation for the observed virtual absence of cross-tolerance following 7 days RJR-2403 treatment is, most likely, is due to the putative desensitization phenomenon that takes place after repeated exposure to nicotine or subtype selective agonists. During the desensitization phase, nAChRs are functionally inactive and do not respond to activation by agonist. This explains the virtual absence of cross-tolerance in animals that received

RJR-2403 treatment for 7 days. Consequently, we did not observe usual RJR-2403's response to antagonize ethanol ataxia.

Cross-Tolerance between Repeated α7 Selective Agonist PNU-282789 and Acute Ethanol Ataxia. Based on our acute behavioral data indicating that PNU-282789 attenuated ethanol ataxia, we sought to explore whether repeated PNU-282789 would also results in the development of tolerance to acute ethanol ataxia. Following the same protocol as was for repeated RJR-2403's exposure, we observed tolerance did indeed develop to ethanol ataxia after PNU-282789 microinfusion. However, the pattern of cross-tolerance between PNU-282789 and ethanol ataxia was distinct from that developed between RJR-2403 and ethanol ataxia. For example, tolerance developed after a single microinfusion of PNU-282789 and lasted for 72 h. We evaluated ethanol ataxia 16 hr following the last microinfusion of PNU-282789 as we did for RJR-2403 to be certain that the parent drug, PNU-282789 would have been eliminated from the animal in this time period. Unlike RJR-2403, the biological half life of PNU-282789 was not available from the literature. Consequently, it was difficult to speculate whether attenuation of ethanol ataxia, 16 h after PNU-282789' infusion represented tolerance or prolonged drug response. As pretreatment with methyllycaconitine, an α_7–subtype selective antagonist, blocked PNU-282789-induced attenuation of ethanol ataxia 16 h after the microinfusion, it was plausible to believe that PNU-282789's action reflected tolerance. This effect could be due to the fact that the biological half life of methyllycaconitine is 20 min in rodents and within 1-2 h methyllycaconitine is completely eliminated from the system **(**Stegelmeier et al., 2003**).** Thus, even if PNU-282789 had a half life longer than 16 h and remained in the tissues long after methyllycaconitine's elimination, it would have attenuated ethanol ataxia afterwards.

However, when microinfusion of PNU-282987 was preceded by methyllycaconitine, the cross-tolerance was not observed indicating that PNU-282987-induced action on α_7 subtype was blocked. This also suggested that the half life of PNU-282987 could not have been long and must be shorter that 16 h. On the contrary, PNU-282789-elicited tolerance required initial activation of the α_7 subtype nAChR and would not occur if receptors were pre-blocked with a selective α_7 antagonist. We also wanted to preclude the possibility of involvement of any non-nicotinic mechanism in the development of the cross-tolerance between PNU-282987 and ethanol ataxia. Therefore, we pretreated animals with hexamethonium followed by PNU-282987 and ethanol. As was observed, hexamethonium pretreatment also abolished the development of cross-tolerance between PNU-282987 and ethanol ataxia thereby confirming that the PNU-282987-induced response was mediated by α_7 subtype of nAChRs only. Like RJR-2403, the phenomenon of receptor desensitization was also observed following repeated treatment. However, unlike RJR-2403, receptor desensitization was seen relatively quicker (i.e. after 3 and 5 days of repeated PNU-282789's microinfusion). As a result of desensitization, there was a significant reduction in the ability of PNU-282789 to attenuate ethanol ataxia. Thus, compared to RJR-2403, PNU-282789-induced desensitization was quicker in onset as supported by others (Alkondon and Albuquerque, 1993; Vibat et al., 1995).

Typically, tolerance represents a gradual cellular adaptive process that requires repeated agonist exposure but in some instances it can develop even with single exposure of an agonist. For example, Zhang et al. (2000) demonstrated the development of acute nicotine tolerance to operant discriminative stimulus in rats and observed the simultaneous upregulation of α_7 subtype in certain brain regions. Our finding of a relatively rapid cross-

tolerance development between α_7 subtype and ethanol ataxia was therefore, in agreement with their report. However, in this case receptor upregulation is less likely to be implicated in cross tolerance as receptor upregulation is generally preceded by receptor desensitization (Spark and Pandy, 1999). Our data identified the occurrence of cross-tolerance even before desensitization set in. Thus, we are inclined to suspect that activation of some common cellular signaling events (i.e. nitric oxide) between nicotinic agonists and ethanol, rather than receptor desensitization or upregulation, as a mechanism in the formation of cross tolerance. In our previous work, we characterized the development of cross-tolerance between nicotine and ethanol (Al-Rejaie and Dar, 2006b,c) and between nicotine, an $\alpha_4\beta_2$ subtype agonist (RJR-2403) and acute ICB Δ^9-tetrahydrocannabinol (Δ^9-THC) in mice (Smith and Dar, 2006).

Localization of $\alpha_4\beta_2$ and α_7 Subtype in Cerebellum

Distribution maps of neuronal nAChR subtypes have been established in several species with receptors composed of the $\alpha_4\beta_2$ subtypes identified as the most abundant and functionally active subtype. The cerebellum is known to express multiple nAChR "α" subtypes including, α_2, α_4, α_5, α_6. However, some controversy exists with respect to the presence of the α_7 subtype nAChR in cerebellum (Caruncho et al., 1997; Gotti and Clementi, 2004; Nashmi and Lester, 2006). Since this dissertation project focused on the study of cerebellar $\alpha_4\beta_2$ and α_7, subtypes, we sought to investigate the distribution of these subtypes in cerebellum to confirm that the behavioral response of selective $\alpha_4\beta_2$ and α_7 agonists is indeed carried out by the respective subtypes and that they are anatomically present. The results of immunohistochemistry experiments revealed that there is moderate expression of both subtypes in the molecular and the Purkinge cell layers with some

sparse distribution in granule cell layer. Such a pattern of distribution of $\alpha_4\beta_2$ and α_7 subtypes correlated with the Rotorod behavioral data. Others have also found α_4 subtype in the Purkinje cell layer, granule cells and the deep cerebellar nuclei while α_7 subtype was more prominently identified in Purkinje cells (Hill et al., 1987; Caruncho et al., 1997; Nakayama et al., 1998), thus corroborating our finding. Thus, the immunohistochemistry data does detects the expression of both α_4 and α_7 subtypes in the cerebellum. The behavioral data, as discussed above demonstrated that both of these nAChR subtypes participate in behavioral responses we observed in the Rotorod test.

Cerebellar Nitric Oxide: Role in Functional Interaction between $\alpha_4\beta_2$ and α_7 selective Drugs and Ethanol Ataxia

Previously, we had elucidated the involvement of cerebellar glutamate-NO-cGMP signaling cascade in the functional interaction between nicotine and acute ethanol- or Δ^9-THC-induced ataxia as well as in the development of cross-tolerance between chronic nicotine and ethanol (Al-Rejaie and Dar, 2006a,b) and between nicotine, its $\alpha_4\beta_2$ subtypes and Δ^9-THC ataxia using pharmacological studies. The result of the pharmacological experiments demonstrated that the ICB microinfusion of NO donors, such as sodium nitroprusside, markedly enhanced the attenuating effects of nicotine on ethanol ataxia. On the other hand, inhibitors of NO such as S-methylisothiourea, significantly decreased the attenuation of ethanol-induced ataxia due to nicotine. The results suggested the involvement of cerebellar NO in nicotine and ethanol functional interactions. Similarly, the activator of cGMP, isoliquiritigenin, microinfused into the cerebellum significantly increased nicotine-induced attenuation of ethanol ataxia. In

contrast, hand, ODQ, an inhibitor of cGMP, markedly decreased nicotine-induced attenuation of ethanol ataxia. The results provided evidence for the involvement of NO-cGMP system in the nicotine and ethanol interaction. Additionally, pretreatment with isoliquirtigenin alone attenuated ethanol ataxia. These results led to the development of the NO-cGMP signaling hypothesis that states that any increase in the activity of NO-cGMP considerably increased the attenuating effect of nicotine as well as that of $\alpha_4\beta_2$ and α_7 subtype agonists, thereby virtually abolishing the ethanol ataxia. Ethanol administration as well as ICB microinfusion of Δ^9-THC markedly inhibited cerebellar NO (Al-Rejaie and Dar, 2006a,b; Smith and Dar, 2007). Therefore, in the present research investigation, to provide the experimental support for the proposed hypothesis, measurement of cerebellar NO was carried out.

In the present study, cerebellar NO_x levels following acute treatment with RJR-2403 and PNU-282987 alone or in the presence of ethanol were measured. The NO data obtained were in general agreement with our hypothesis (i.e., the functional interaction between nicotine and ethanol is modulated by cerebellar NO-cGMP signaling). The nicotinic subtype agonists, RJR-2403, PNU-282987, each significantly elevated NO_x levels compared to aCSF control while acute ethanol administration significantly diminished cerebellar NO_x concentration (Fig. 29). These changes in the cerebellar NO_x levels functionally correlate very well with ethanol- induced ataxia and its antagonism by selective nAChR subtype agonists. In other words, the decrease in cerebellar NO_x levels by ethanol correlated with the observed ethanol ataxia. Whereas the marked attenuation of ethanol ataxia by ICB pretreatment with RJR-2403 and PNU-282987 associated well with an increase in NO_x level due to RJR-2403 and PNU-282987 and/or with their opposition to the

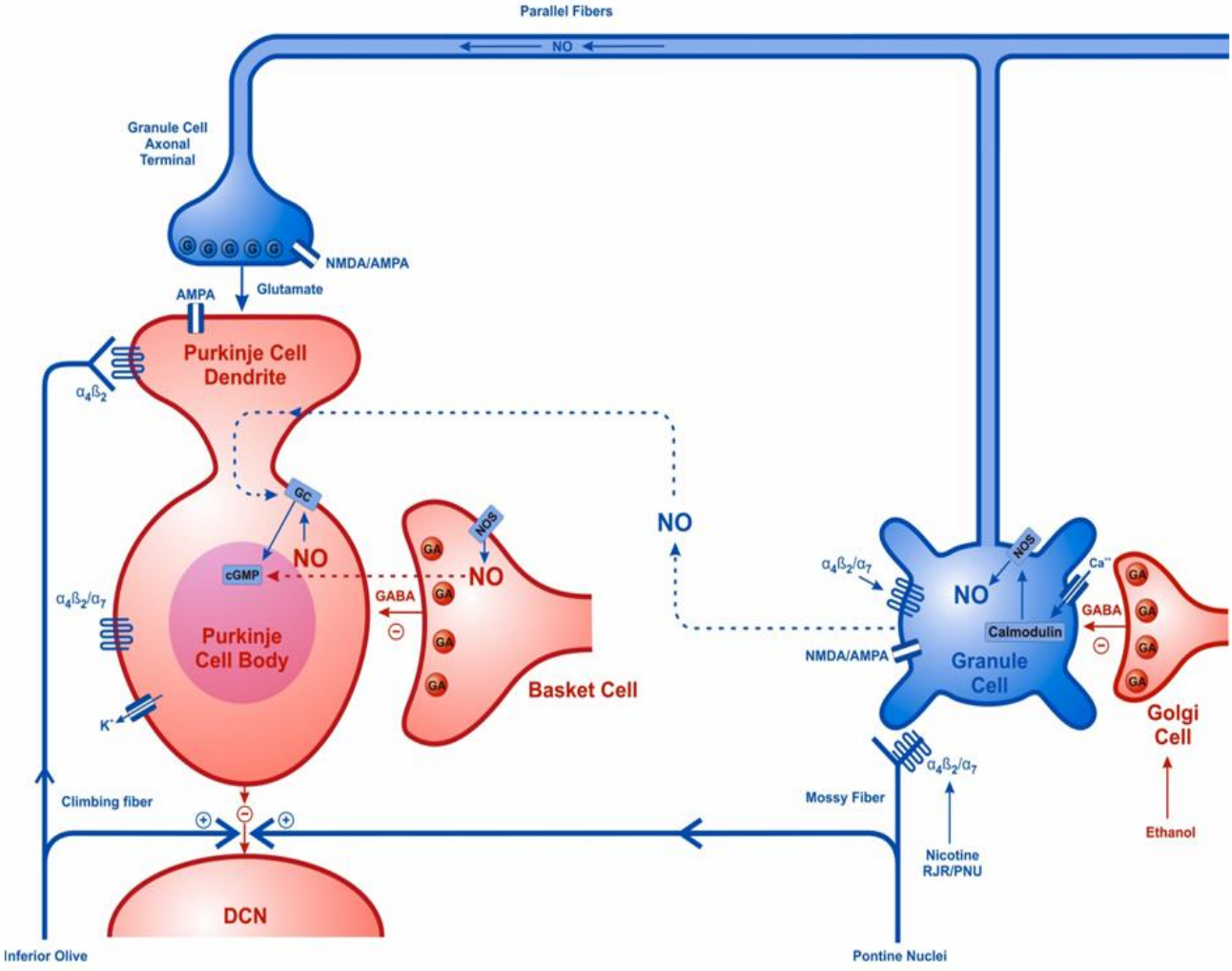
NO_x lowering effect of acute ethanol. Similar data was obtained with repeated RJR-2403 and PNU-282987 microinfusion (i.e. elevation of cerebellar NOx levels that persisted at least 16 h after the last agonist micoinfusion [Fig. 30]). Chronically elevated levels of NOx significantly reversed the acute ethanol-induced fall in NOx levels leading to restoration of normal motor coordination. Thus, it was also implied that NO signaling participates in the mediation of cross-tolerance between RJR-2403 or PNU-282987 and ethanol ataxia. This observation was identical to previous work where repeated ICB nicotine also elevated cerebellar NOx levels that pertained to attenuation of ethanol ataxia (Al-Rejaie and Dar, 2006a,b).

The effect of ethanol on NOS activity is variable with regards to dose and site of action (Chandler et al. 1994). According to some reports, ethanol decreases NO release and inhibits NOS activity (Chandler et al., 1994; Czapski et al., 2002). These observations suggest that a strong relationship may exist between NO-cGMP cascade and regulation of motor coordination in cerebellum. Cerebellar NOS is located in granule and basket cells where NO is released to act on Purkinje cells (Fedele et al., 1999). Both Purkinje and granule cells, exhibit strong immunoreactivity for guanylyl cyclase (Ding et al., 2004). Thus, NO would stimulate cGMP production in Purkinje cells resulting in decreased Purkinje cell firing. Purkinje cells represent the only inhibitory output in the cerebellum and any event leading to depression of Purkinje cell firing would cause a decrease in GABAergic transmission within the Purkinje cells. Similarly, cellular changes might have occurred following nicotine/RJR-2403/PNU-282987 microinfusion as α_7 nAChR receptors are reportedly located at the mossy cell-granule cell synapse where nicotinic receptor agonists mediate glutamate release (Reno et al., 2004).

Cellular Mechanisms between Nicotine-Ethanol Functional Interaction

The motor coordination activities are regulated by a delicate balance between GABAergic inhibitory and glutamatergic excitatory signals operating within the cerebellar cortical granule and Purkinje cells, respectively, and any disruption this balance can lead to loss of motor control. To help explain the underlying mechanism for the functional nicotine and ethanol interaction, we suggest that nicotine/RJR-2403/PNU-282987 and ethanol modulate excitatory and inhibitory signals in the cerebellum, respectively. The observed ethanol-induced motor incoordination and the attenuation of motor incoordination by intracerebellar RJR-2403/PNU-282987 microinfusion was the net outcome of whether inhibitory or excitatory signals are dominant. Ethanol is known to potentiate GABAergic transmission by stimulating Golgi cell firing within the cerebellum (Carta et al., 2004) (Fig. 32). The GABA release at the Golgi cell-granule cell synapses depresses the firing of granule cells which represent the only excitatory neuronal cells within the cerebellum (Voogd and Glickstein, 1998). In the absence of adequate excitatory signals this balance is shifted in favor of inhibitory signals producing inhibition of Purkinje cells. Therefore, deep cerebellar nuclei (DCN), the major output neurons in the cerebellum undergo profound inhibition which will result in motor incoordination (i.e., ataxia). The excitatory neurotransmitter glutamate is released by nicotine/RJR-2403/PNU-282987 to significantly enhance the firing rate of granule cells (Fig. 32). The parallel fibers of the granule cells synapse extensively with the dendritic tree of Purkinje cells. Purkinje cells express voltage-gated K^+ channels and the activation of Purkinje cells activates K^+ channels which promotes an outward flow of K^+ ion and hyperpolarization (Yazdi et al., 2007; Womack et al., 2009). The net effect will be a substantially reduced firing of Purkinje cells. Purkinje

FIGURE 32: Proposed schematic diagram of the effects of RJR-2403/PNU-282987 on the attenuation of ethanol ataxia in the cerebellar cortex.


Parallel Fibers
NO
Granule Cell Axonal Terminal
NMDA/AMPA
AMPA
Glutamate
Purkinje Cell Dendrite
$\alpha_4\beta_2$
GC
NO
cGMP
Purkinje Cell Body
$\alpha_4\beta_2/\alpha_7$
K+
NOS
NO
GABA
GA
Basket Cell
NO
$\alpha_4\beta_2/\alpha_7$
NOS
NO
Ca++
Calmodulin
NMDA/AMPA
Granule Cell
GABA
GA
Golgi Cell
Ethanol
$\alpha_4\beta_2/\alpha_7$
Mossy Fiber
Nicotine RJR/PNU
Climbing fiber
DCN
Inferior Olive
Pontine Nuclei

cells are the largest inhibitory neurons in cerebellum and function to discharge inhibitory output to deep cerebellar nuclei (DCN). Once Purkinje cell-induced inhibitory transmission is depressed, the DCN are disinhibited while granule cell-mediated firing will be unopposed to further increase rate. The overall effect would be that DCN will fire at a higher rate than before which will normalize aberrant motor activities.

Our results implicate the significance of cerebellar cortex for the action of nicotinic receptor compounds to overcome ethanol-induced ataxia, which is mediated by multiple sites in the brain including the cerebellum (Al-Rejaie and Dar, 2006a,b), the striatum (Meng and Dar, 1996) and the motor cortex (Barwick and Dar, 1998). Based on the functional mapping of the cerebellum the site of action of nicotine lies within -6.0 to -6.4 AP stereotaxic coordinates in the cerebellar cortex as nicotine's microinfusion beyond this range had no effect on ethanol ataxia. It is fascinating to propose that ICB microinfusion of nicotine to such a small site in the anterior lobe (culmen area) of the cerebellum exerts such a powerful anti-ethanol effect. Ethanol was always administered systemically (i.p.) and therefore, distributed to entire body including the whole brain. There are at least two other brain areas (striatum and motor cortex) that are known to mediate ethanol-induced ataxia, yet ICB microinfusion of nicotine virtually abolished the ataxia due to ethanol.

Selectivity of the Drugs Used in this Project

For the purpose of this project, agents that were highly selective for nicotinic $\alpha_4\beta_2$ and α_7 subtypes were selected to characterize the role of those respective subtype(s) in the nicotine-ethanol interaction. For example, RJR-2403 (Fig. 33), a highly selective and potent $\alpha_4\beta_2$ receptor agonist, has Ki values of 26nM for $\alpha_4\beta_2$ and 3.6µM for α_7 subtype. RJR is less potent than nicotine in inducing dopamine release from synpatosomes and in

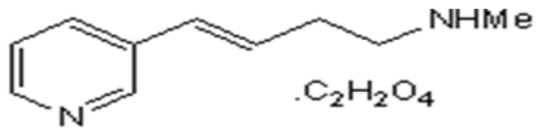
inducing ileum contraction. However, the compound is equipotent to nicotine in improving working and reference memory and in activating rat thalamic synaptosomes (Bencherif et al., 1996) and less potent in decreasing body temperature, respiration, and increasing heart rate and BP (Lippiello et al., 1996). Also, RJR-2403 is more selective for central than peripheral $\alpha_4\beta_2$ subtypes.

DHβE (Fig. 33) is a short acting competitive antagonist with selectivity for $\alpha_4\beta_2$ subtypes (ki 109nM) (Shafaee et al., 1999) and effectively prevented several actions of nicotine including, antinociception, hypolocomotion, motor impairment, hypothermia and discriminative stimulus effects but failed to inhibit nicotine-induced seizures in mice (Damaj et al., 1995a,b, 1999).

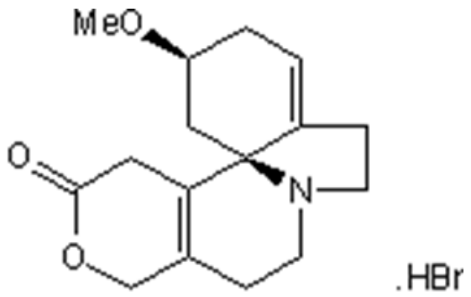
PNU-282987 (Fig. 34) is a potent, α_7-selective agonist (Ki= 26nM) with low activity at $\alpha_1\beta_3$ and $\alpha_3\beta_3$ nAChR subtypes (Ki> 60μM). In addition, the compound possesses virtually no activity on monoamine, muscarinic, glutamate and GABa receptors greater 1μM. However, PNU-282987 does display some affinity for $5HT_3$ receptors (Ki=930nM) (Bodnar et al., 2005). In hippocampal slices, PNU-282987 evoked whole cell currents that were counteracted by methyllycaconitine. Activation of α_7 transiently increases CREB and ERK phosphorylation, which appears to be a critical step required for long term memory consolidation (Bitner et al., 2007).

Methyllycaconitine (Fig. 34), a natural norditerpenoid alkaloid, is a reversible competitive antagonist for homomeric α_7 subtype. Methyllycaconitine displays about 100 fold selectivity for α_7 (ki= 1.4 nM) than non- α_7 subtype (Ward et al., 1990). Compared to muscle α_7 subtype, neuronal α_7 subtype has high affinity for methyllycaconitine. Inhibition to α_7 subtype, by methyllycaconitine, precipitated

FIGURE 33: Chemical structures of RJR-2403, a selective $\alpha_4\beta_2$ nAChR subtype agonist, and DHβE, a selective $\alpha_4\beta_2$ selective antagonist



RJR-2403 oxalate [(E)-N-Methyl-4-(3-pyridinyl)-3-buten-1-amine oxalate]

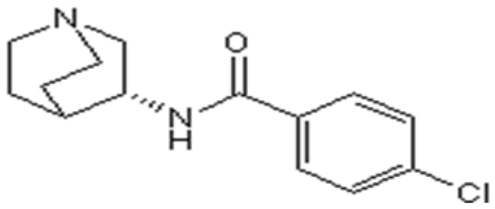


Dihydro-β-erythroidine hydrobromide (DHβE) [(2S,13bS)-2-Methoxy-2,3,5,6,8,9,10,13-octahydro-1H,12H- benzo[i]pyrano[3,4-g]indolizin-12-one hydrobromide]

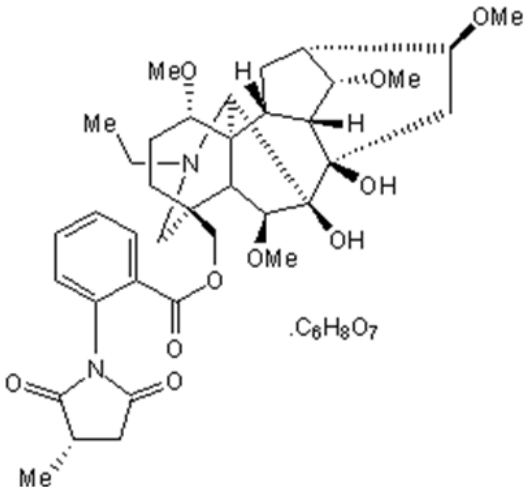
withdrawal responses in mice chronically exposed to nicotine (Salas et a., 2007) and increased cocaine induced dopamine release in nucleus accumbens. When microinfused into the frontal cortex, methyllycaconitine disrupted both working and reference memory performance while DHβE impaired working memory only (Chan et al., 2007).

Most pharmacological agents are known to exhibit selective affinity for certain receptors or subtypes within a certain dose range. Beyond that range their action might spill over to other related receptor types. To address this issue and to ensure the selectivity of our selected doses for this project, we designed an experiment in which mice were pretreated with α_7-subtype selective antagonist, methyllycaconitine, followed by treatment with the $\alpha_4\beta_2$ selective agonist, RJR-2403, and then ethanol. As shown in Fig. 16, methyllycaconitine did not block RJR2403-induced attenuation of ethanol ataxia indicating that the action of RJR-2403 was probably carried out by $\alpha_4\beta_2$ subtype since methyllycaconitine would have prevented had it been mediated by α_7 subtype, This was also inferred that methyllycaconitine at 6ng displays selective α_7 blockade because the same methyllycaconitine dose (6ng) completely eliminated the action of PNU-282987 2.5µg (Fig. 15). We also believe that PNU-282987 92.5µg) exhibited selective α_7 response only. However, there was no statistically significant difference between the pharmacological response to PNU-282987 2.5 µg and PNU-282987 250 ng in acute studies. We continued further experiments with PNU-282987 250ng. On the other hand methyllycaconitine 12 ng, interrupted RJR's action revealing the loss of selectivity at this very dose.

FIGURE 34: Chemical structures of PNU-282987, a selective α_7 nAChR subtype agonist, and methyllycaconitine, a selective α_7 selective antagonist



PNU 282987 [N-(3R)-1-Azabicyclo[2.2.2]oct-3-yl-4-chlorobenzamide]



Methyllycaconitine citrate (MLA) [1a,4(S),6b,14a,16b]-20-Ethyl-1,6,14,16-tetramethoxy-4- [[[2-(3-methyl-2,5- dioxo-1-pyrrolidinyl)benzoyl]oxy]methyl]aconitane-7,8-diol citrate]

Clinical Significance of Research

The present studies demonstrated a functional antagonistic interaction between the two most widely consumed psychoactive drugs. The observation of nicotine-induced attenuation of ethanol ataxia, and the development of cross tolerance between $\alpha_4\beta_2$ and α_7 selective agonists and acute ethanol ataxia provides a clue as to why the co-addiction or co-dependence occurs between nicotine and ethanol. By extrapolating the current animal data to human system it could be speculated that the reduction of ethanol-induce impairment in motor coordination or cognition by simultaneous smoking provides a motivation for the drinkers to further drink followed by more smoking. This trend rapidly builds dependence to both nicotine and ethanol. Current clinical protocols for the treatment of alcoholism or smoking alone have low success rates and are hampered by high relapse rates due to the mutual dependence on both drugs. This requires thorough understanding of the neurobiology of co-addiction as well as the functional interaction between nicotine and ethanol to identify novel therapeutic targets. This research projects delineates the contribution of nAChR subtypes ($\alpha_4\beta_2$, α_7) in the mediation of functional interaction between nicotine and ethanol. Based on the findings of these studies, it is proposed that the pharmacological maninpulation of $\alpha_4\beta_2$ and α_7 subtypes could dissociate the functional relationship between the two drugs and could provide a treatment for co-addiction. Moreover, data from this project suggest that selective agonists at nAChR subtypes $\alpha_4\beta_2$ and α_7 can be used to design future treatments for ethanol ataxia, which accounts for significant number of traffic accidental deaths and disabilities.

Conclusion

The present studies demonstrated that the nicotine-induced attenuation of ethanol ataxia is mediated by cerebellar nAChR subtypes $\alpha_4\beta_2$ and α_7. Selective agonists at $\alpha_4\beta_2$ and α_7 subtypes decreased while their antagonists did not change ethanol ataxia. The studies also demonstrated the development of cross-tolerance between $\alpha_4\beta_2$ and α_7 selective agonists and acute ethanol ataxia in repeated treatment paradigm. Both subtypes contributed equally significantly in nicotine's reduction of ethanol ataxia, yet there are subtype-related differences in the onset and duration of the cross tolerance as well as the desensitization of the subtypes. While $\alpha_4\beta_2$-subtype-mediated cross-tolerance was slow in onset and shorter in duration, the α_7 subtype-induced cros-tolerance developed rapidly and lasted longer. The nitric oxide-cGMP signaling was found to be a key regulator of the functional antagonism detected between nicotinic drugs and ethanol. The cerebellar nitrate+nitrite (measured as total tissue nitrite; NO_x) levels augmented with nicotinic subtype agonists and decreased with ethanol, indicating, attenuation and induction of ataxia, respectively. Consistent with our behavioral and biochemical data, both $\alpha_4\beta_2$ and α_7 subtypes were found in moderate density in cerebellar cortex, with $\alpha_4\beta_2$ expression slightly higher than that of the α_7. Findings from this project provide further insight into an antagonistic relationship between nicotine and ethanol and help explain as to how this functional interaction may form the basis for co-addiction.

Future Directions

This research projects investigates an important aspect of nicotine-ethanol's functional interaction that is mediated through cerebellar $\alpha_4\beta_2$ and α_7 subtypes and

that could possibly be a potential underlying factor for co-addiction. In addition to cerebellum, striatum and motor cortex are the key brain areas critically involved in the regulation of motor coordination. Therefore, future studies might be designed to explore if nAChR subtypes $\alpha_4\beta_2$ and α_7 in these brain regions mediate nicotine-ethanol interaction. A recent report by Gotti et al., (2008) has demonstrated the role of $\alpha_6\beta_2$ subtype in nicotine-induced hyperlocomotor and dopamine rewarding action, implying the possible involvement of $\alpha_6\beta_2$ subtype in reversing the ethanol ataxia. Since moderate levels of $\alpha_6\beta_2$ subtypes are also expressed in cerebellum, it might be interesting to see if pharmacological manipulation of $\alpha_6\beta_2$ subtype alters nicotine-ethanol's antagonistic interaction in cerebellum or other areas. Moreover, the present studies were based on the hypothesis that nicotine-induced reduction of ethanol ataxia might be a contributing factor for co-addiction. However, the project was not extended to observe any correlation between ethanol-evoked ataxic phase and increase in nicotine self- administration. Any demonstration of this type of positive correlation in animal model will further strengthen the current hypothesis.

Finally, this project determined the alterations in nitric oxide levels following nicotinic drugs or ethanol administration while the expression levels for NOS were not measured. It would be interesting to see if NOS enzyme undergo upregulation following chronic nicotinic agonist treatment.

REFERENCES

Addy NA, Nakijama A and Levin ED (2003) Nicotinic mechanisms of memory: effects of acute local DHbetaE and MLA infusions in the basolateral amygdala. *Brain Res Cogn Brain Res* **16**:51-57.

Aistrup GL, Marszalec W and Narahashi T (1999) Ethanol modulation of nicotinic acetylcholine receptor currents in cultured cortical neurons. *Mol Pharmacol* **55**:39-49.

Alkondon M and Albuquerque EX (1993) Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons. I. Pharmacological and functional evidence for distinct structural subtypes. *J Pharmacol Exp Ther* **265**:1455-1473.

Al-Rejaie S and Dar MS (2006a) Antagonism of ethanol ataxia by intracerebellar nicotine: possible modulation by mouse cerebellar nitric oxide and cGMP. *Brain Res Bull* **69**:187-196.

Al-Rejaie S and Dar MS (2006b) Behavioral interaction between nicotine and ethanol: possible modulation by mouse cerebellar glutamate. *Alcohol Clin Exp Res* **30**:1223-1233.

Al-Rejaie S and Dar MS (2006c) Possible role of mouse cerebellar nitric oxide in the behavioral interaction between chronic intracerebellar nicotine and acute ethanol administration: observation of cross-tolerance. *Neuroscience* **138**:575-585.

Alvestad RM, Grosshans DR, Coultrap SJ, Nakazawa T, Yamamoto T and Browning MD (2003) Tyrosine dephosphorylation and ethanol inhibition of N-Methyl-D-aspartate receptor function. *J Biol Chem* **278**:11020-11025.

Anders DL, Blevins T, Sutton G, Chandler LJ and Woodward JJ (1999) Effects of c-Src tyrosine kinase on ethanol sensitivity of recombinant NMDA receptors expressed in HEK 293 cells. *Alcohol Clin Exp Res* **23**:357-362.

Anthony JC and Echeagaray-Wagner F (2000) Epidemiologic analysis of alcohol and tobacco use. *Alcohol Res Health* **24**:201-208.

Anwer J and Dar MS (1995) In vivo effects of (-)-nicotine on ethanol-induced increase in glucose utilization in the mouse cerebellum. *Brain Res Bull* **36**:343-348.

Aubert I, Araujo DM, Cecyre D, Robitaille Y, Gauthier S and Quirion R (1992) Comparative alterations of nicotinic and muscarinic binding sites in Alzheimer's and Parkinson's diseases. *J Neurochem* **58**:529-541.

Ayajiki K, Hayashida H, Okamura T and Toda N (1997) Pelvic nerve stimulation-induced pressor responses in corpus cavernosum of anesthetized dogs. *Am J Physiol* **273**:H2141-2145.

Balfour D, Bates C, Benowitz N, Berridge V, Britton J, Callum C, Channer K, Cuthberstson L, Foulds J, Godfrey C, Hajek P, Henningfield JC, Hughes JR, Jarvis M, McNeil A, Owen L, Raw M, Sandford A, Slade J, Stapleton J, Stolerman I, Sutherland G, Sweanor D, West R, Wonnacott S (2000) Nicotine addiction in brain. A report of the Tobacco Advisory Group of the Royal College of Physicians. London: ISBN

Balfour DJ, Benwell ME, Birrell CE, Kelly RJ and Al-Aloul M (1998) Sensitization of the mesoaccumbens dopamine response to nicotine. *Pharmacol Biochem Behav* **59**:1021-1030.

Barwick VS and Dar MS (1998) Adenosinergic modulation of ethanol-induced motor incoordination in the rat motor cortex. *Prog Neuropsychopharmacol Biol Psychiatry* **22**:587-607.

Bate L, Gardiner M (1999) Structure of a neuronal nicotinic acetylcholine receptor (nAChR). Expert Reviews in Molecular Medicine Cambridge University Press Cambridge.

Beani L, Bianchi C, Nilsson L, Nordberg A, Romanelli L and Sivilotti L (1985) The effect of nicotine and cytisine on 3H-acetylcholine release from cortical slices of guinea-pig brain. *Naunyn Schmiedebergs Arch Pharmacol* **331**:293-296.

Beckman JS, Beckman TW, Chen J, Marshall PA and Freeman BA (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A* **87**:1620-1624.

Belluardo N, Mudo G, Blum M, Amato G and Fuxe K (2000) Neurotrophic effects of central nicotinic receptor activation. *J Neural Transm Suppl*:227-245.

Bencherif M, Lovette ME, Fowler KW, Arrington S, Reeves L, Caldwell WS and Lippiello PM (1996) RJR-2403: a nicotinic agonist with CNS selectivity I. In vitro characterization. *J Pharmacol Exp Ther* **279**:1413-1421.

Bianchi C, Tomasini MC, Antonelli T, Marani L and Beani L (2000) Nicotinic modulation of [(3)H]D-aspartate outflow from cultured cerebellar granule cells. *Synapse* **36**:307-313.

Bitner RS, Bunnelle WH, Anderson DJ, Briggs CA, Buccafusco J, Curzon P, Decker MW, Frost JM, Gronlien JH, Gubbins E, Li J, Malysz J, Markosyan S, Marsh K, Meyer MD, Nikkel AL, Radek RJ, Robb HM, Timmermann D, Sullivan JP and Gopalakrishnan M (2007) Broad-spectrum efficacy across cognitive domains by

alpha7 nicotinic acetylcholine receptor agonism correlates with activation of ERK1/2 and CREB phosphorylation pathways. *J Neurosci* **27**:10578-10587.

Bitner RS, Nikkel AL, Curzon P, Donnelly-Roberts DL, Puttfarcken PS, Namovic M, Jacobs IC, Meyer MD and Decker MW (2000) Reduced nicotinic receptor-mediated antinociception following in vivo antisense knock-down in rat. *Brain Res* **871**:66-74.

Bodnar I, Banky Z, Nagy GM and Halasz B (2005) Non-NMDA glutamate receptor antagonist injected into the hypothalamic paraventricular nucleus blocks the suckling stimulus-induced release of prolactin. *Brain Res Bull* **65**:163-168.

Bonthius DJ, McKim RA, Koele L, Harb H, Kehrberg AH, Mahoney J, Karacay B and Pantazis NJ (2006) Severe alcohol-induced neuronal deficits in the hippocampus and neocortex of neonatal mice genetically deficient for neuronal nitric oxide synthase (nNOS). *J Comp Neurol* **499**:290-305.

Botta P, Radcliffe RA, Carta M, Mameli M, Daly E, Floyd KL, Deitrich RA and Valenzuela CF (2007) Modulation of GABAA receptors in cerebellar granule neurons by ethanol: a review of genetic and electrophysiological studies. *Alcohol* **41**:187-199.

Bowers BJ, Elliott KJ and Wehner JM (2001) Differential sensitivity to the anxiolytic effects of ethanol and flunitrazepam in PKCgamma null mutant mice. *Pharmacol Biochem Behav* **69**:99-110.

Bowers BJ, McClure-Begley TD, Keller JJ, Paylor R, Collins AC and Wehner JM (2005) Deletion of the alpha7 nicotinic receptor subunit gene results in increased sensitivity to several behavioral effects produced by alcohol. *Alcohol Clin Exp Res* **29**:295-302.

Brandon NJ, Delmas P, Kittler JT, McDonald BJ, Sieghart W, Brown DA, Smart TG and Moss SJ (2000) GABAA receptor phosphorylation and functional modulation in cortical neurons by a protein kinase C-dependent pathway. *J Biol Chem* **275**:38856-38862.

Breese CR, Marks MJ, Logel J, Adams CE, Sullivan B, Collins AC and Leonard S (1997) Effect of smoking history on [3H]nicotine binding in human postmortem brain. *J Pharmacol Exp Ther* **282**:7-13.

Broide RS, Salas R, Ji D, Paylor R, Patrick JW, Dani JA and De Biasi M (2002) Increased sensitivity to nicotine-induced seizures in mice expressing the L250T alpha 7 nicotinic acetylcholine receptor mutation. *Mol Pharmacol* **61**:695-705.

Buisson B and Bertrand D (2002) Nicotine addiction: the possible role of functional upregulation. *Trends Pharmacol Sci* **23**:130-136.

Calabrese V, Mancuso C, Calvani M, Rizzarelli E, Butterfield DA and Stella AM (2007) Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity. *Nat Rev Neurosci* **8**:766-775.

Calapai G and Caputi AP (1996) Nitric oxide and drinking behaviour. *Regul Pept* **66**:117-121.

Camarini R and Hodge CW (2004) Ethanol preexposure increases ethanol self-administration in C57BL/6J and DBA/2J mice. *Pharmacol Biochem Behav* **79**:623-632.

Caputo F, Trevisani F and Bernardi M (2007) Alcohol misuse and traffic accidents. *Lancet* **369**:463-464.

Carta M, Mameli M and Valenzuela CF (2004) Alcohol enhances GABAergic transmission to cerebellar granule cells via an increase in Golgi cell excitability. *J Neurosci* **24**:3746-3751.

Caruncho HJ, Guidotti A, Lindstrom J, Costa E and Pesold C (1997) Subcellular localization of the alpha 7 nicotinic receptor in rat cerebellar granule cell layer. *Neuroreport* **8**:1431-1433.

Ceballos NA (2006) Tobacco use, alcohol dependence, and cognitive performance. *J Gen Psychol* **133**:375-388.

Chan WK, Wong PT and Sheu FS (2007) Frontal cortical alpha7 and alpha4beta2 nicotinic acetylcholine receptors in working and reference memory. *Neuropharmacology* **52**:1641-1649.

Chandler LJ, Guzman NJ, Sumners C and Crews FT (1994) Magnesium and zinc potentiate ethanol inhibition of N-methyl-D-aspartate-stimulated nitric oxide synthase in cortical neurons. *J Pharmacol Exp Ther* **271**:67-75.

Chandler LJ, Sutton G, Norwood D, Sumners C and Crews FT (1997) Chronic ethanol increases N-methyl-D-aspartate-stimulated nitric oxide formation but not receptor density in cultured cortical neurons. *Mol Pharmacol* **51**:733-740.

Changeux JP, Bertrand D, Corringer PJ, Dehaene S, Edelstein S, Lena C, Le Novere N, Marubio L, Picciotto M and Zoli M (1998) Brain nicotinic receptors: structure and regulation, role in learning and reinforcement. *Brain Res Brain Res Rev* **26**:198-216.

Cho CH, Song W, Leitzell K, Teo E, Meleth AD, Quick MW and Lester RA (2005) Rapid upregulation of alpha7 nicotinic acetylcholine receptors by tyrosine dephosphorylation. *J Neurosci* **25**:3712-3723.

Choi DS, Wei W, Deitchman JK, Kharazia VN, Lesscher HM, McMahon T, Wang D, Qi ZH, Sieghart W, Zhang C, Shokat KM, Mody I and Messing RO (2008) Protein kinase Cdelta regulates ethanol intoxication and enhancement of GABA-stimulated tonic current. *J Neurosci* **28**:11890-11899.

Choi DS, Wei W, Deitchman JK, Kharazia VN, Lesscher HM, McMahon T, Wang D, Qi ZH, Sieghart W, Zhang C, Shokat KM, Mody I and Messing RO (2008) Protein kinase Cdelta regulates ethanol intoxication and enhancement of GABA-stimulated tonic current. *J Neurosci* **28**:11890-11899.

Clarke PB (1990) Mesolimbic dopamine activation--the key to nicotine reinforcement? *Ciba Found Symp* **152**:153-162; discussion 162-158.

Clarke PB, Schwartz RD, Paul SM, Pert CB and Pert A (1985) Nicotinic binding in rat brain: autoradiographic comparison of [3H]acetylcholine, [3H]nicotine, and [125I]-alpha-bungarotoxin. *J Neurosci* **5**:1307-1315.

Cohen C, Bergis OE, Galli F, Lochead AW, Jegham S, Biton B, Leonardon J, Avenet P, Sgard F, Besnard F, Graham D, Coste A, Oblin A, Curet O, Voltz C, Gardes A, Caille D, Perrault G, George P, Soubrie P and Scatton B (2003) SSR591813, a novel selective and partial alpha4beta2 nicotinic receptor agonist with potential as an aid to smoking cessation. *J Pharmacol Exp Ther* **306**:407-420.

Collins AC, Wilkins LH, Slobe BS, Cao JZ and Bullock AE (1996) Long-term ethanol and nicotine treatment elicit tolerance to ethanol. *Alcohol Clin Exp Res* **20**:990-999.

Cook JB, Foster KL, Eiler WJ, 2nd, McKay PF, Woods J, 2nd, Harvey SC, Garcia M, Grey C, McCane S, Mason D, Cummings R, Li X, Cook JM and June HL (2005) Selective GABAA alpha5 benzodiazepine inverse agonist antagonizes the neurobehavioral actions of alcohol. *Alcohol Clin Exp Res* **29**:1390-1401.

Cooper RG and Magwere T (2008) Nitric oxide-mediated pathogenesis during nicotine and alcohol consumption. *Indian J Physiol Pharmacol* **52**:11-18.

Corringer PJ, Le Novere N and Changeux JP (2000) Nicotinic receptors at the amino acid level. *Annu Rev Pharmacol Toxicol* **40**:431-458.

Court JA, Martin-Ruiz C, Graham A and Perry E (2000) Nicotinic receptors in human brain: topography and pathology. *J Chem Neuroanat* **20**:281-298.

Covernton PJ and Connolly JG (1997) Differential modulation of rat neuronal nicotinic receptor subtypes by acute application of ethanol. *Br J Pharmacol* **122**:1661-1668.

Cui C, Booker TK, Allen RS, Grady SR, Whiteaker P, Marks MJ, Salminen O, Tritto T, Butt CM, Allen WR, Stitzel JA, McIntosh JM, Boulter J, Collins AC and Heinemann SF (2003) The beta3 nicotinic receptor subunit: a component of alpha-conotoxin

MII-binding nicotinic acetylcholine receptors that modulate dopamine release and related behaviors. *J Neurosci* **23**:11045-11053.

Curzon P, Brioni JD and Decker MW (1996) Effect of intraventricular injections of dihydro-beta-erythroidine (DH beta E) on spatial memory in the rat. *Brain Res* **714**:185-191.

Czapski GA, Sun GY and Strosznajder JB (2002) Inhibition of N-methyl-D-aspartic acid-nitric oxide synthase in rat hippocampal slices by ethanol. Evidence for the involvement of tetrahydrobiopterin but not lipid peroxidation. *J Biomed Sci* **9**:3-9.

Dajas-Bailador FA, Mogg AJ and Wonnacott S (2002) Intracellular Ca2+ signals evoked by stimulation of nicotinic acetylcholine receptors in SH-SY5Y cells: contribution of voltage-operated Ca2+ channels and Ca2+ stores. *J Neurochem* **81**:606-614.

Damaj MI, Creasy KR, Welch SP, Rosecrans JA, Aceto MD and Martin BR (1995a) Comparative pharmacology of nicotine and ABT-418, a new nicotinic agonist. *Psychopharmacology (Berl)* **120**:483-490.

Damaj MI, Glassco W, Dukat M and Martin BR (1999) Pharmacological characterization of nicotine-induced seizures in mice. *J Pharmacol Exp Ther* **291**:1284-1291.

Damaj MI, Welch SP and Martin BR (1995b) In vivo pharmacological effects of dihydro-beta-erythroidine, a nicotinic antagonist, in mice. *Psychopharmacology (Berl)* **117**:67-73.

Dani JA (2001) Overview of nicotinic receptors and their roles in the central nervous system. *Biol Psychiatry* **49**:166-174.

Dani JA and Bertrand D (2007) Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. *Annu Rev Pharmacol Toxicol* **47**:699-729.

Dani JA, Ji D and Zhou FM (2001) Synaptic plasticity and nicotine addiction. *Neuron* **31**:349-352.

Dar MS (1988) The biphasic effects of centrally and peripherally administered caffeine on ethanol-induced motor incoordination in mice. *J Pharm Pharmacol* **40**:482-487.

Dar MS (1990) Functional correlation between subclasses of brain adenosine receptor affinities and ethanol-induced motor incoordination in mice. *Pharmacol Biochem Behav* **37**:747-753.

Dar MS (1997) Mouse cerebellar adenosinergic modulation of ethanol-induced motor incoordination: possible involvement of cAMP. *Brain Res* **749**:263-274.

Dar MS and Meng ZH (2004) Acute ethanol-induced adenosine diphosphate ribosylation regulates the functional activity of rat striatal pertussis toxin-sensitive g proteins. *Alcohol Clin Exp Res* **28**:1299-1307.

Dar MS, Bowman ER and Li C (1994) Intracerebellar nicotinic-cholinergic participation in the cerebellar adenosinergic modulation of ethanol-induced motor incoordination in mice. *Brain Res* **644**:117-127.

Dar MS, Li C and Bowman ER (1993) Central behavioral interactions between ethanol, (-)-nicotine, and (-)-cotinine in mice. *Brain Res Bull* **32**:23-28.

Dar MS, Mustafa SJ and Wooles WR (1983) Possible role of adenosine in the CNS effects of ethanol. *Life Sci* **33**:1363-1374.

de Fiebre CM and Collins AC (1993) A comparison of the development of tolerance to ethanol and cross-tolerance to nicotine after chronic ethanol treatment in long- and short-sleep mice. *J Pharmacol Exp Ther* **266**:1398-1406.

De Fiebre CM, Medhurst LJ and Collins AC (1987) Nicotine response and nicotinic receptors in long-sleep and short-sleep mice. *Alcohol* **4**:493-501.

De Filippi G, Baldwinson T and Sher E (2005) Nicotinic receptor modulation of neurotransmitter release in the cerebellum. *Prog Brain Res* **148**:307-320.

Decker DA and Galligan JJ (2009) Cross-inhibition between nicotinic acetylcholine receptors and P2X receptors in myenteric neurons and HEK-293 cells. *Am J Physiol Gastrointest Liver Physiol* **296**:G1267-1276.

Decker MW, Sullivan JP, Arneric SP and Williams M (1999) Neuronal nicotinic acetylcholine receptors: Novel targets for CNS therapeutics, in *Psychopharmacology: Fourth Generation of Progress CD ROM Version ACNP*, Lippincott Williams & Wilkins, Baltimore, MD.

Diamond I and Gordon AS (1994) The role of adenosine in mediating cellular and molecular responses to ethanol. *EXS* **71**:175-183.

Dickinson JA, Hanrott KE, Mok MH, Kew JN and Wonnacott S (2007) Differential coupling of alpha7 and non-alpha7 nicotinic acetylcholine receptors to calcium-induced calcium release and voltage-operated calcium channels in PC12 cells. *J Neurochem* **100**:1089-1096.

DiFranza JR and Guerrera MP (1990) Alcoholism and smoking. *J Stud Alcohol* **51**:130-135.

Ding X, Li Z, Yang J, Hou JG and Zhu Q (2004) Theoretical study of nitric oxide adsorption on Au clusters. *J Chem Phys* **121**:2558-2562.

Dohrman DP and Reiter CK (2003) Ethanol modulates nicotine-induced upregulation of nAChRs. *Brain Res* **975**:90-98.

Dopico AM, Anantharam V and Treistman SN (1998) Ethanol increases the activity of Ca(++)-dependent K+ (mslo) channels: functional interaction with cytosolic Ca++. *J Pharmacol Exp Ther* **284**:258-268.

Dwoskin LP, Wooters TE, Sumithran SP, Siripurapu KB, Joyce BM, Lockman PR, Manda VK, Ayers JT, Zhang Z, Deaciuc AG, McIntosh JM, Crooks PA and Bardo MT (2008) N,N'-Alkane-diyl-bis-3-picoliniums as nicotinic receptor antagonists: inhibition of nicotine-evoked dopamine release and hyperactivity. *J Pharmacol Exp Ther* **326**:563-576.

Eilers H, Schaeffer E, Bickler PE and Forsayeth JR (1997) Functional deactivation of the major neuronal nicotinic receptor caused by nicotine and a protein kinase C-dependent mechanism. *Mol Pharmacol* **52**:1105-1112.

Elgoyhen AB, Johnson DS, Boulter J, Vetter DE and Heinemann S (1994) Alpha 9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. *Cell* **79**:705-715.

Faccidomo S, Besheer J, Stanford PC and Hodge CW (2009) Increased operant responding for ethanol in male C57BL/6J mice: specific regulation by the ERK1/2, but not JNK, MAP kinase pathway. *Psychopharmacology (Berl)* **204**:135-147.

Fataccioli V, Gentil M, Nordmann R and Rouach H (1997) Inactivation of cerebellar nitric oxide synthase by ethanol in vitro. *Alcohol Alcohol* **32**:683-691.

Fedele E and Raiteri M (1999) In vivo studies of the cerebral glutamate receptor/NO/cGMP pathway. *Prog Neurobiol* **58**:89-120.

Fedele E, Varnier G, Ansaldo MA and Raiteri M (1998) Nicotine administration stimulates the in vivo N-methyl-D-aspartate receptor/nitric oxide/cyclic GMP pathway in rat hippocampus through glutamate release. *Br J Pharmacol* **125**:1042-1048.

Federici M, Nistico R, Giustizieri M, Bernardi G and Mercuri NB (2009) Ethanol enhances GABAB-mediated inhibitory postsynaptic transmission on rat midbrain dopaminergic neurons by facilitating GIRK currents. *Eur J Neurosci* **29**:1369-1377.

Fenster CP, Whitworth TL, Sheffield EB, Quick MW and Lester RA (1999) Upregulation of surface alpha4beta2 nicotinic receptors is initiated by receptor desensitization after chronic exposure to nicotine. *J Neurosci* **19**:4804-4814.

Ferchmin PA, Perez D, Eterovic VA and de Vellis J (2003) Nicotinic receptors differentially regulate N-methyl-D-aspartate damage in acute hippocampal slices. *J Pharmacol Exp Ther* **305**:1071-1078.

Fernandez-Cancio M, Fernandez-Vitos EM, Centelles JJ and Imperial S (2001) Sources of interference in the use of 2,3-diaminonaphthalene for the fluorimetric determination of nitric oxide synthase activity in biological samples. *Clin Chim Acta* **312**:205-212.

Flores CM, Davila-Garcia MI, Ulrich YM and Kellar KJ (1997) Differential regulation of neuronal nicotinic receptor binding sites following chronic nicotine administration. *J Neurochem* **69**:2216-2219.

Franceschini D, Paylor R, Broide R, Salas R, Bassetto L, Gotti C, De Biasi M (2002) Absence of alpha7-containing neuronal nicotinic acetylcholine receptors does not prevent nicotine-induced seizures.Brain Res Mol Brain Res, 98:29-40

French SJ, Humby T, Horner CH, Sofroniew MV and Rattray M (1999) Hippocampal neurotrophin and trk receptor mRNA levels are altered by local administration of nicotine, carbachol and pilocarpine. *Brain Res Mol Brain Res* **67**:124-136.

Galzi JL and Changeux JP (1995) Neuronal nicotinic receptors: molecular organization and regulations. *Neuropharmacology* **34**:563-582.

Gately I (2001) Tobacco: A cultural history of how an exotic plant seduced civilization. Grove Press: New York City, New York, USA

Gentry CL and Lukas RJ (2002) Regulation of nicotinic acetylcholine receptor numbers and function by chronic nicotine exposure. *Curr Drug Targets CNS Neurol Disord* **1**:359-385.

Ghalayini IF (2004) Nitric oxide-cyclic GMP pathway with some emphasis on cavernosal contractility. *Int J Impot Res* **16**:459-469.

Girod R, Barazangi N, McGehee D and Role LW (2000) Facilitation of glutamatergic neurotransmission by presynaptic nicotinic acetylcholine receptors. *Neuropharmacology* **39**:2715-2725.

Gopalakrishnan M, Monteggia LM, Anderson DJ, Molinari EJ, Piattoni-Kaplan M, Donnelly-Roberts D, Arneric SP and Sullivan JP (1996) Stable expression, pharmacologic properties and regulation of the human neuronal nicotinic acetylcholine alpha 4 beta 2 receptor. *J Pharmacol Exp Ther* **276**:289-297.

Gorbounova O, Svensson AL, Jonsson P, Mousavi M, Miao H, Hellstrom-Lindahl E and Nordberg A (1998) Chronic ethanol treatment decreases [3H]epibatidine and [3H]nicotine binding and differentially regulates mRNA levels of nicotinic acetylcholine receptor subunits expressed in M10 and SH-SY5Y neuroblastoma cells. *J Neurochem* **70**:1134-1142.

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

Gotti C, Fornasari D and Clementi F (1997) Human neuronal nicotinic receptors. *Prog Neurobiol* **53**:199-237.

Gotti C, Guiducci S, Tedesco V, Corbioli S, Zanetti L, Moretti M, Zanardi A, Rimondini R, Mugnaini M, Clementi F, Chiamulera C and Zoli M Nicotinic acetylcholine receptors in the mesolimbic pathway: primary role of ventral tegmental area alpha6beta2* receptors in mediating systemic nicotine effects on dopamine release, locomotion, and reinforcement. *J Neurosci* **30**:5311-5325.

Gotti C, Hanke W, Maury K, Moretti M, Ballivet M, Clementi F and Bertrand D (1994) Pharmacology and biophysical properties of alpha 7 and alpha 7-alpha 8 alpha-bungarotoxin receptor subtypes immunopurified from the chick optic lobe. *Eur J Neurosci* **6**:1281-1291.

Graham A, Court JA, Martin-Ruiz CM, Jaros E, Perry R, Volsen SG, Bose S, Evans N, Ince P, Kuryatov A, Lindstrom J, Gotti C and Perry EK (2002) Immunohistochemical localisation of nicotinic acetylcholine receptor subunits in human cerebellum. *Neuroscience* **113**:493-507.

Gulick D and Gould TJ (2008) Interactive effects of ethanol and nicotine on learning in C57BL/6J mice depend on both dose and duration of treatment. *Psychopharmacology (Berl)* **196**:483-495.

Harris RA (1999) Ethanol actions on multiple ion channels: which are important? *Alcohol Clin Exp Res* **23**:1563-1570.

Haustein KO (2003) Tobacco or Health? Physiological and social damages caused by tobacco smoking. Springer

Haycock JW (1993) Multiple forms of tyrosine hydroxylase in human neuroblastoma cells: quantitation with isoform-specific antibodies. *J Neurochem* **60**:493-502.

Hays JT, Schroeder DR, Offord KP, Croghan IT, Patten CA, Hurt RD, Jorenby DE and Fiore MC (1999) Response to nicotine dependence treatment in smokers with current and past alcohol problems. *Ann Behav Med* **21**:244-250.

Henningfield JE and Keenan RM (1993) Nicotine delivery kinetics and abuse liability. *J Consult Clin Psychol* **61**:743-750.

Herber DL, Severance EG, Cuevas J, Morgan D and Gordon MN (2004) Biochemical and histochemical evidence of nonspecific binding of alpha7nAChR antibodies to mouse brain tissue. *J Histochem Cytochem* **52**:1367-1376.

Hills JM, Jessen KR and Mirsky R (1987) An immunohistochemical study of the distribution of enteric GABA-containing neurons in the rat and guinea-pig intestine. *Neuroscience* **22**:301-312.

Hindmarch I, Kerr JS and Sherwood N (1990) Effects of nicotine gum on psychomotor performance in smokers and non-smokers. *Psychopharmacology (Berl)* **100**:535-541.

Ho KK and Flood P (2004) Single amino acid residue in the extracellular portion of transmembrane segment 2 in the nicotinic alpha7 acetylcholine receptor modulates sensitivity to ketamine. *Anesthesiology* **100**:657-662.

Hong SK, Jung IS, Bang SA and Kim SE (2006) Effect of nitric oxide synthase inhibitor and NMDA receptor antagonist on the development of nicotine sensitization of nucleus accumbens dopamine release: an in vivo microdialysis study. *Neurosci Lett* **409**:220-223.

Hsu YN, Amin J, Weiss DS and Wecker L (1996) Sustained nicotine exposure differentially affects alpha 3 beta 2 and alpha 4 beta 2 neuronal nicotinic receptors expressed in Xenopus oocytes. *J Neurochem* **66**:667-675.

Hu D, Cao K, Peterson-Wakeman R and Wang R (2002) Altered profile of gene expression in rat hearts induced by chronic nicotine consumption. *Biochem Biophys Res Commun* **297**:729-736.

Ikeda M, Komiyama T, Sato I, Himi T and Murota S (1999) Neuronal nitric oxide synthase is resistant to ethanol. *Life Sci* **64**:1623-1630.

Iorio KR, Reinlib L, Tabakoff B and Hoffman PL (1992) Chronic exposure of cerebellar granule cells to ethanol results in increased N-methyl-D-aspartate receptor function. *Mol Pharmacol* **41**:1142-1148.

Itzhak Y and Anderson KL (2008) Ethanol-induced behavioral sensitization in adolescent and adult mice: role of the nNOS gene. *Alcohol Clin Exp Res* **32**:1839-1848.

Itzhak Y and Martin JL (2002) Effect of the neuronal nitric oxide synthase inhibitor 7-nitroindazole on methylphenidate-induced hyperlocomotion in mice. *Behav Pharmacol* **13**:81-86.

Jang MH, Lee MH, Kim H, Lee SJ, Sim YJ, Kim CJ, Park SK, Kim J and Kim EH (2005) Maternal alcohol administration suppresses expression of nitric oxide synthase in the hippocampus of offspring rats. *J Pharmacol Sci* **98**:459-462.

Jia Y, Yamazaki Y, Nakauchi S and Sumikawa K (2009) Alpha2 nicotine receptors function as a molecular switch to continuously excite a subset of interneurons in rat hippocampal circuits. *Eur J Neurosci* **29**:1588-1603.

Jones GM, Sahakian BJ, Levy R, Warburton DM and Gray JA (1992) Effects of acute subcutaneous nicotine on attention, information processing and short-term memory in Alzheimer's disease. *Psychopharmacology (Berl)* **108**:485-494.

Karlin A (1993) Structure of nicotinic acetylcholine receptors. *Curr Opin Neurobiol* **3**:299-309.

Kelm MK, Criswell HE and Breese GR (2008) The role of protein kinase A in the ethanol-induced increase in spontaneous GABA release onto cerebellar Purkinje neurons. *J Neurophysiol* **100**:3417-3428.

Kenny PJ, Chartoff E, Roberto M, Carlezon WA, Jr. and Markou A (2009) NMDA receptors regulate nicotine-enhanced brain reward function and intravenous nicotine self-administration: role of the ventral tegmental area and central nucleus of the amygdala. *Neuropsychopharmacology* **34**:266-281.

Kihara T, Shimohama S, Urushitani M, Sawada H, Kimura J, Kume T, Maeda T and Akaike A (1998) Stimulation of alpha4beta2 nicotinic acetylcholine receptors inhibits beta-amyloid toxicity. *Brain Res* **792**:331-334.

Kishi M and Steinbach JH (2006) Role of the agonist binding site in up-regulation of neuronal nicotinic alpha4beta2 receptors. *Mol Pharmacol* **70**:2037-2044.

Knowles RG and Moncada S (1994) Nitric oxide synthases in mammals. *Biochem J* **298 (Pt 2)**:249-258.

Kobayashi T, Ikeda K, Kojima H, Niki H, Yano R, Yoshioka T and Kumanishi T (1999) Ethanol opens G-protein-activated inwardly rectifying K+ channels. *Nat Neurosci* **2**:1091-1097.

Konu O, Kane JK, Barrett T, Vawter MP, Chang R, Ma JZ, Donovan DM, Sharp B, Becker KG and Li MD (2001) Region-specific transcriptional response to chronic nicotine in rat brain. *Brain Res* **909**:194-203.

Korkosz A, Zatorski P, Taracha E, Plaznik A, Kostowski W and Bienkowski P (2006a) Ethanol blocks nicotine-induced seizures in mice: comparison with midazolam and baclofen. *Alcohol* **40**:151-157.

Korkosz A, Zatorski P, Taracha E, Plaznik A, Kostowski W and Bienkowski P (2006b) Effects of ethanol on nicotine-induced conditioned place preference in C57BL/6J mice. *Prog Neuropsychopharmacol Biol Psychiatry* **30**:1283-1290.

Kumar S, Lane BM and Morrow AL (2006) Differential effects of systemic ethanol administration on protein kinase cepsilon, gamma, and beta isoform expression,

membrane translocation, and target phosphorylation: reversal by chronic ethanol exposure. *J Pharmacol Exp Ther* **319**:1366-1375.

Kuper H, Adami HO and Boffetta P (2002) Tobacco use, cancer causation and public health impact. *J Intern Med* **251**:455-466.

Laitinen JT, Laitinen KS, Tuomisto L and Airaksinen MM (1994) Differential regulation of cyclic GMP levels in the frontal cortex and the cerebellum of anesthetized rats by nitric oxide: an in vivo microdialysis study. *Brain Res* **668**:117-121.

Lallemand F, Ward RJ, Dravolina O and De Witte P (2006) Nicotine-induced changes of glutamate and arginine in naive and chronically alcoholized rats: an in vivo microdialysis study. *Brain Res* **1111**:48-60.

Lapin EP, Maker HS and Bhardwaj A (1995) Ethanol enhancement of the motor-stimulating effect of nicotine in the rat. *Alcohol* **12**:217-220.

Larsson A and Engel JA (2004) Neurochemical and behavioral studies on ethanol and nicotine interactions. *Neurosci Biobehav Rev* **27**:713-720.

Larsson A, Svensson L, Soderpalm B and Engel JA (2002) Role of different nicotinic acetylcholine receptors in mediating behavioral and neurochemical effects of ethanol in mice. *Alcohol* **28**:157-167.

Laudenbach V, Medja F, Zoli M, Rossi FM, Evrard P, Changeux JP and Gressens P (2002) Selective activation of central subtypes of the nicotinic acetylcholine receptor has opposite effects on neonatal excitotoxic brain injuries. *FASEB J* **16**:423-425.

Lawrence AD and Sahakian BJ (1998) The cognitive psychopharmacology of Alzheimer's disease: focus on cholinergic systems. *Neurochem Res* **23**:787-794.

Le AD, Corrigall WA, Harding JW, Juzytsch W, Li TK (2000) Involvement of nicotinic receptors in alcohol self-administration. Alcohol Clin Exp Res 24:155-63

Levin ED (2002) Nicotinic receptor subtypes and cognitive function. *J Neurobiol* **53**:633-640.

Levin ED and Simon BB (1998) Nicotinic acetylcholine involvement in cognitive function in animals. *Psychopharmacology (Berl)* **138**:217-230.

Levin ED and Torry D (1996) Acute and chronic nicotine effects on working memory in aged rats. *Psychopharmacology (Berl)* **123**:88-97.

Levin ED, Bettegowda C, Blosser J and Gordon J (1999) AR-R17779, an alpha 7 nicotinic agonist, improves learning and memory in rats. *Behavioural Pharmacology* **10**:675-680.

Levin ED, McClernon FJ and Rezvani AH (2006) Nicotinic effects on cognitive function: behavioral characterization, pharmacological specification, and anatomic localization. *Psychopharmacology (Berl)* **184**:523-539.

Lewohl JM, Wilson WR, Mayfield RD, Brozowski SJ, Morrisett RA and Harris RA (1999) G-protein-coupled inwardly rectifying potassium channels are targets of alcohol action. *Nat Neurosci* **2**:1084-1090.

Li C, Peoples RW and Weight FF (1998) Ethanol-induced inhibition of a neuronal P2X purinoceptor by an allosteric mechanism. *Br J Pharmacol* **123**:1-3.

Lippiello PM, Bencherif M, Gray JA, Peters S, Grigoryan G, Hodges H and Collins AC (1996) RJR-2403: a nicotinic agonist with CNS selectivity II. In vivo characterization. *J Pharmacol Exp Ther* **279**:1422-1429.

Littleton J, Barron S, Prendergast M and Nixon SJ (2007) Smoking kills (alcoholics)! shouldn't we do something about it? *Alcohol Alcohol* **42**:167-173.

Lopez-Hernandez GY, Sanchez-Padilla J, Ortiz-Acevedo A, Lizardi-Ortiz J, Salas-Vincenty J, Rojas LV and Lasalde-Dominicci JA (2004) Nicotine-induced up-regulation and desensitization of alpha4beta2 neuronal nicotinic receptors depend on subunit ratio. *J Biol Chem* **279**:38007-38015.

Ma XL, Li YH, Gao JX, Li J, Guo L and Wu CZ (2008) Expression of inducible nitric oxide synthase in the liver is under the control of nuclear factor kappa B in concanavalin A-induced hepatitis. *J Gastroenterol Hepatol* **23**:e231-235.

MacDermott AB, Role LW and Siegelbaum SA (1999) Presynaptic ionotropic receptors and the control of transmitter release. *Annu Rev Neurosci* **22**:443-485.

Maffei A, Prestori F, Shibuki K, Rossi P, Taglietti V and D'Angelo E (2003) NO enhances presynaptic currents during cerebellar mossy fiber-granule cell LTP. *J Neurophysiol* **90**:2478-2483.

Mai H, May WS, Gao F, Jin Z and Deng X (2003) A functional role for nicotine in Bcl2 phosphorylation and suppression of apoptosis. *J Biol Chem* **278**:1886-1891.

Majchrzak MJ and Dilsaver SC (1992) Chronic treatment with ethanol alters the physiological action of nicotine. *Prog Neuropsychopharmacol Biol Psychiatry* **16**:107-115.

Marks MJ, Pauly JR, Gross SD, Deneris ES, Hermans-Borgmeyer I, Heinemann SF and Collins AC (1992) Nicotine binding and nicotinic receptor subunit RNA after chronic nicotine treatment. *J Neurosci* **12**:2765-2784.

Marshall DL, Redfern PH and Wonnacott S (1997) Presynaptic nicotinic modulation of dopamine release in the three ascending pathways studied by in vivo microdialysis: comparison of naive and chronic nicotine-treated rats. *J Neurochem* **68**:1511-1519.

Marubio LM, del Mar Arroyo-Jimenez M, Cordero-Erausquin M, Lena C, Le Novere N, de Kerchove d'Exaerde A, Huchet M, Damaj MI and Changeux JP (1999) Reduced antinociception in mice lacking neuronal nicotinic receptor subunits. *Nature* **398**:805-810.

McDonald BJ and Moss SJ (1997) Conserved phosphorylation of the intracellular domains of GABA(A) receptor beta2 and beta3 subunits by cAMP-dependent protein kinase, cGMP-dependent protein kinase protein kinase C and Ca2+/calmodulin type II-dependent protein kinase. *Neuropharmacology* **36**:1377-1385.

McGehee DS and Role LW (1995) Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. *Annu Rev Physiol* **57**:521-546.

McIntosh JM, Absalom N, Chebib M, Elgoyhen AB and Vincler M (2009) Alpha9 nicotinic acetylcholine receptors and the treatment of pain. *Biochem Pharmacol* **78**:693-702.

McKay BE, Placzek AN and Dani JA (2007) Regulation of synaptic transmission and plasticity by neuronal nicotinic acetylcholine receptors. *Biochem Pharmacol* **74**:1120-1133.

Melis M, Camarini R, Ungless MA and Bonci A (2002) Long-lasting potentiation of GABAergic synapses in dopamine neurons after a single in vivo ethanol exposure. *J Neurosci* **22**:2074-2082.

Meng ZH and Dar MS (1996) Dispersion characteristics of [3H]-labeled adenosine agonist/antagonist following their intrastriatal microinfusion. *Methods Find Exp Clin Pharmacol* **18**:373-386.

Meyerhoff DJ, Tizabi Y, Staley JK, Durazzo TC, Glass JM, Nixon SJ (2006) Smoking comorbidity in alcoholism: neurobiological and neurocognitive consequences. Alcohol Clin Exp Res 30:253-64.

Mokdad AH, Marks JS, Stroup DF and Gerberding JL (2004) Actual causes of death in the United States, 2000. *JAMA* **291**:1238-1245.

Molinari EJ, Delbono O, Messi ML, Renganathan M, Arneric SP, Sullivan JP and Gopalakrishnan M (1998) Up-regulation of human alpha7 nicotinic receptors by chronic treatment with activator and antagonist ligands. *Eur J Pharmacol* **347**:131-139.

Morris SM, Jr. (2005) Arginine metabolism in vascular biology and disease. *Vasc Med* **10 Suppl 1**:S83-87.

Moykkynen T, Korpi ER and Lovinger DM (2003) Ethanol inhibits alpha-amino-3-hydyroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor function in central nervous system neurons by stabilizing desensitization. *J Pharmacol Exp Ther* **306**:546-555.

Mudo G, Belluardo N and Fuxe K (2007) Nicotinic receptor agonists as neuroprotective/neurotrophic drugs. Progress in molecular mechanisms. *J Neural Transm* **114**:135-147.

Nagata K, Aistrup GL, Huang CS, Marszalec W, Song JH, Yeh JZ and Narahashi T (1996) Potent modulation of neuronal nicotinic acetylcholine receptor-channel by ethanol. *Neurosci Lett* **217**:189-193.

Nagy LE, Diamond I and Gordon AS (1991) cAMP-dependent protein kinase regulates inhibition of adenosine transport by ethanol. *Mol Pharmacol* **40**:812-817.

Nakayama H, Shioda S, Nakajo S, Ueno S and Nakai Y (1998) Expression of the nicotinic acetylcholine receptor alpha4 subunit mRNA in the rat cerebellar cortex. *Neurosci Lett* **256**:177-179.

Nakayama H, Shioda S, Nakajo S, Ueno S, Nakashima T and Nakai Y (1997) Immunocytochemical localization of nicotinic acetylcholine receptor in the rat cerebellar cortex. *Neurosci Res* **29**:233-239.

Narahashi T, Aistrup GL, Marszalec W and Nagata K (1999) Neuronal nicotinic acetylcholine receptors: a new target site of ethanol. *Neurochem Int* **35**:131-141.

Narahashi T, Soderpalm B, Ericson M, Olausson P, Engel JA, Zhang X, Nordberg A, Marszalec W, Aistrup GL, Schmidt LG, Kalouti U, Smolka and Hedlund L (2001) Mechanisms of alcohol-nicotine interactions: alcoholics versus smokers. *Alcohol Clin Exp Res* **25**:152S-156S.

Nashmi R and Lester HA (2006) CNS localization of neuronal nicotinic receptors. *J Mol Neurosci* **30**:181-184.

Nashmi R, Xiao C, Deshpande P, McKinney S, Grady SR, Whiteaker P, Huang Q, McClure-Begley T, Lindstrom JM, Labarca C, Collins AC, Marks MJ and Lester HA (2007) Chronic nicotine cell specifically upregulates functional alpha 4* nicotinic receptors: basis for both tolerance in midbrain and enhanced long-term potentiation in perforant path. *J Neurosci* **27**:8202-8218.

Neugebauer NM, Zhang Z, Crooks PA, Dwoskin LP and Bardo MT (2006) Effect of a novel nicotinic receptor antagonist, N,N'-dodecane-1,12-diyl-bis-3-picolinium

dibromide, on nicotine self-administration and hyperactivity in rats. *Psychopharmacology (Berl)* **184**:426-434.

Newton PM and Messing RO (2006) Intracellular signaling pathways that regulate behavioral responses to ethanol. *Pharmacol Ther* **109**:227-237.

Nguyen HN, Rasmussen BA and Perry DC (2004) Binding and functional activity of nicotinic cholinergic receptors in selected rat brain regions are increased following long-term but not short-term nicotine treatment. *J Neurochem* **90**:40-49.

Nott A and Levin ED (2006) Dorsal hippocampal alpha7 and alpha4beta2 nicotinic receptors and memory. *Brain Res* **1081**:72-78.

Olale F, Gerzanich V, Kuryatov A, Wang F and Lindstrom J (1997) Chronic nicotine exposure differentially affects the function of human alpha3, alpha4, and alpha7 neuronal nicotinic receptor subtypes. *J Pharmacol Exp Ther* **283**:675-683.

Papke RL (1993) The kinetic properties of neuronal nicotinic receptors: genetic basis of functional diversity. *Prog Neurobiol* **41**:509-531.

Paradiso KG and Steinbach JH (2003) Nicotine is highly effective at producing desensitization of rat alpha4beta2 neuronal nicotinic receptors. *J Physiol* **553**:857-871.

Paterson D and Nordberg A (2000) Neuronal nicotinic receptors in the human brain. *Prog Neurobiol* **61**:75-111.

Peng X, Gerzanich V, Anand R, Whiting PJ and Lindstrom J (1994) Nicotine-induced increase in neuronal nicotinic receptors results from a decrease in the rate of receptor turnover. *Mol Pharmacol* **46**:523-530.

Pereira BJ, King AJ, Poutsiaka DD, Strom JA and Dinarello CA (1993) Comparison of first use and reuse of cuprophan membranes on interleukin-1 receptor antagonist and interleukin-1 beta production by blood mononuclear cells. *Am J Kidney Dis* **22**:288-295.

Perry DC, Xiao Y, Nguyen HN, Musachio JL, Davila-Garcia MI and Kellar KJ (2002) Measuring nicotinic receptors with characteristics of alpha4beta2, alpha3beta2 and alpha3beta4 subtypes in rat tissues by autoradiography. *J Neurochem* **82**:468-481.

Picciotto MR (2003) Nicotine as a modulator of behavior: beyond the inverted U. *Trends Pharmacol Sci* **24**:493-499.

Picciotto MR and Corrigall WA (2002) Neuronal systems underlying behaviors related to nicotine addiction: neural circuits and molecular genetics. *J Neurosci* **22**:3338-3341.

Picciotto MR, Zoli M, Rimondini R, Lena C, Marubio LM, Pich EM, Fuxe K and Changeux JP (1998) Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. *Nature* **391**:173-177.

Pogun S, Demirgoren S, Taskiran D, Kanit L, Yilmaz O, Koylu EO, Balkan B and London ED (2000) Nicotine modulates nitric oxide in rat brain. *Eur Neuropsychopharmacol* **10**:463-472.

Pomerleau CS, Aubin HJ and Pomerleau OF (1997) Self-reported alcohol use patterns in a sample of male and female heavy smokers. *J Addict Dis* **16**:19-24.

Pontieri FE, Tanda G, Orzi F and Di Chiara G (1996) Effects of nicotine on the nucleus accumbens and similarity to those of addictive drugs. *Nature* **382**:255-257.

Rao MR, Hutcheson AE and Markov AK (1998) Changes in the cardiovascular nitric oxide pathway in cyclosporin-A treated rats. *Drug Chem Toxicol* **21**:27-34.

Rassoulpour A, Wu HQ, Albuquerque EX and Schwarcz R (2005) Prolonged nicotine administration results in biphasic, brain-specific changes in kynurenate levels in the rat. *Neuropsychopharmacology* **30**:697-704.

Reno LA, Zago W and Markus RP (2004) Release of [(3)H]-L-glutamate by stimulation of nicotinic acetylcholine receptors in rat cerebellar slices. *Neuroscience* **124**:647-653.

Rezvani AH and Levin ED (2002) Nicotine-alcohol interactions and cognitive function in rats. *Pharmacol Biochem Behav* **72**:865-872.

Riganti L, Matteoni C, Di Angelantonio S, Nistri A, Gaimarri A, Sparatore F, Canu-Boido C, Clementi F and Gotti C (2005) Long-term exposure to the new nicotinic antagonist 1,2-bisN-cytisinylethane upregulates nicotinic receptor subtypes of SH-SY5Y human neuroblastoma cells. *Br J Pharmacol* **146**:1096-1109.

Robello M, Amico C, Bucossi G, Cupello A, Rapallino MV and Thellung S (1996) Nitric oxide and GABAA receptor function in the rat cerebral cortex and cerebellar granule cells. *Neuroscience* **74**:99-105.

Robinson SE, Vann RE, Britton AF, O'Connell MM, James JR and Rosecrans JA (2007) Cellular nicotinic receptor desensitization correlates with nicotine-induced acute behavioral tolerance in rats. *Psychopharmacology (Berl)* **192**:71-78.

Roca DJ, Rozenberg I, Farrant M and Farb DH (1990) Chronic agonist exposure induces down-regulation and allosteric uncoupling of the gamma-aminobutyric acid/benzodiazepine receptor complex. *Mol Pharmacol* **37**:37-43.

Rodvelt KR, Bumgarner DM, Putnam WC and Miller DK (2007) WIN-55,212-2 and SR-141716A alter nicotine-induced changes in locomotor activity, but do not alter nicotine-evoked [3H]dopamine release. *Life Sci* **80**:337-344.

Romberger DJ and Grant K (2004) Alcohol consumption and smoking status: the role of smoking cessation. *Biomed Pharmacother* **58**:77-83.

Room R (2004) Smoking and drinking as complementary behaviours. *Biomed Pharmacother* **58**:111-115.

Ross SA, Wong JY, Clifford JJ, Kinsella A, Massalas JS, Horne MK, Scheffer IE, Kola I, Waddington JL, Berkovic SF and Drago J (2000) Phenotypic characterization of an alpha 4 neuronal nicotinic acetylcholine receptor subunit knock-out mouse. *J Neurosci* **20**:6431-6441.

Rousseau SJ, Jones IW, Pullar IA and Wonnacott S (2005) Presynaptic alpha7 and non-alpha7 nicotinic acetylcholine receptors modulate [3H]d-aspartate release from rat frontal cortex in vitro. *Neuropharmacology* **49**:59-72.

Ryan RE, Ross SA, Drago J and Loiacono RE (2001) Dose-related neuroprotective effects of chronic nicotine in 6-hydroxydopamine treated rats, and loss of neuroprotection in alpha4 nicotinic receptor subunit knockout mice. *Br J Pharmacol* **132**:1650-1656.

Salas R, Main A, Gangitano D and De Biasi M (2007) Decreased withdrawal symptoms but normal tolerance to nicotine in mice null for the alpha7 nicotinic acetylcholine receptor subunit. *Neuropharmacology* **53**:863-869.

Sastry BV (1995) Neuropharmacology of nicotine: effects on the autoregulation of acetylcholine release by substance P and methionine enkephalin in rodent cerebral slices and toxicological implications. *Clin Exp Pharmacol Physiol* **22**:288-290.

Schrattenholz A, Pereira EF, Roth U, Weber KH, Albuquerque EX and Maelicke A (1996) Agonist responses of neuronal nicotinic acetylcholine receptors are potentiated by a novel class of allosterically acting ligands. *Mol Pharmacol* **49**:1-6.

Schuckit MA (1994) Alcohol sensitivity and dependence. *EXS* **71**:341-348.

Shafaee N, Houng M, Truong A, Viseshakul N, Figl A, Sandhu S, Forsayeth JR, Dwoskin LP, Crooks PA and Cohen BN (1999) Pharmacological similarities between native brain and heterologously expressed alpha4beta2 nicotinic receptors. *Br J Pharmacol* **128**:1291-1299.

Singer S, Rossi S, Verzosa S, Hashim A, Lonow R, Cooper T, Sershen H and Lajtha A (2004) Nicotine-induced changes in neurotransmitter levels in brain areas associated with cognitive function. *Neurochem Res* **29**:1779-1792.

Slotnick BM and Leonard CM (1975) *A Stereotaxic Atlas of Albino Mouse Forebrain,* United States Government Printing Office, Washington, DC.

Smith AD and Dar MS (2006) Mouse cerebellar nicotinic-cholinergic receptor modulation of Delta9-THC ataxia: role of the alpha4beta2 subtype. *Brain Res* **1115**:16-25.

Smith AD and Dar MS (2007) Behavioral cross-tolerance between repeated intracerebellar nicotine and acute Delta(9)-tetrahydrocannabinol-induced cerebellar ataxia: role of cerebellar nitric oxide. *J Pharmacol Exp Ther* **322**:243-253.

Snell LD, Tabakoff B and Hoffman PL (1993) Radioligand binding to the N-methyl-D-aspartate receptor/ionophore complex: alterations by ethanol in vitro and by chronic in vivo ethanol ingestion. *Brain Res* **602**:91-98.

Snyder SH (1994) Nitric oxide. More jobs for that molecule. *Nature* **372**:504-505.

Söderpalm B, Ericson M, Olausson P, Blomqvist O, Engel JA (2000) Nicotinic mechanisms involved in the dopamine activating and reinforcing properties of ethanol. Behav Brain Res 113:85-96 Review

Solinas M, Scherma M, Fattore L, Stroik J, Wertheim C, Tanda G, Fratta W and Goldberg SR (2007) Nicotinic alpha 7 receptors as a new target for treatment of cannabis abuse. *J Neurosci* **27**:5615-5620.

Spanagel R, Siegmund S, Cowen M, Schroff KC, Schumann G, Fiserova M, Sillaber I, Wellek S, Singer M and Putzke J (2002) The neuronal nitric oxide synthase gene is critically involved in neurobehavioral effects of alcohol. *J Neurosci* **22**:8676-8683.

Sparks JA and Pauly JR (1999) Effects of continuous oral nicotine administration on brain nicotinic receptors and responsiveness to nicotine in C57Bl/6 mice. *Psychopharmacology (Berl)* **141**:145-153.

Stachowiak MK, Moffett J, Joy A, Puchacz E, Florkiewicz R and Stachowiak EK (1994) Regulation of bFGF gene expression and subcellular distribution of bFGF protein in adrenal medullary cells. *J Cell Biol* **127**:203-223.

Steensland P, Simms JA, Holgate J, Richards JK and Bartlett SE (2007) Varenicline, an alpha4beta2 nicotinic acetylcholine receptor partial agonist, selectively decreases ethanol consumption and seeking. *Proc Natl Acad Sci U S A* **104**:12518-12523.

Stegelmeier BL, Hall JO, Gardner DR and Panter KE (2003) The toxicity and kinetics of larkspur alkaloid, methyllycaconitine, in mice. *J Anim Sci* **81**:1237-1241.

Stuehr DJ and Griffith OW (1992) Mammalian nitric oxide synthases. *Adv Enzymol Relat Areas Mol Biol* **65**:287-346.

Svensson AL and Nordberg A (1996) Tacrine interacts with an allosteric activator site on alpha 4 beta 2 nAChRs in M10 cells. *Neuroreport* **7**:2201-2205.

Swanson LW, Simmons DM, Whiting PJ and Lindstrom J (1987) Immunohistochemical localization of neuronal nicotinic receptors in the rodent central nervous system. *J Neurosci* **7**:3334-3342.

Tanobe Y, Okamura T, Fujimura M and Toda N (1995) Functional role and histological demonstration of nitric-oxide-mediated inhibitory nerves in dog sphincter of Oddi. *Neurogastroenterol Motil* **7**:219-227.

Taslim N, Al-Rejaie S and Saeed Dar M (2008) Attenuation of ethanol-induced ataxia by alpha(4)beta(2) nicotinic acetylcholine receptor subtype in mouse cerebellum: a functional interaction. *Neuroscience* **157**:204-213.

Tehrani MH and Barnes EM, Jr. (1997) Sequestration of gamma-aminobutyric acidA receptors on clathrin-coated vesicles during chronic benzodiazepine administration in vivo. *J Pharmacol Exp Ther* **283**:384-390.

Tizabi Y, Copeland RL, Jr., Louis VA and Taylor RE (2002a) Effects of combined systemic alcohol and central nicotine administration into ventral tegmental area on dopamine release in the nucleus accumbens. *Alcohol Clin Exp Res* **26**:394-399.

Tizabi Y, Manaye KF, Taylor RE (2005) Nicotine blocks ethanol-induced apoptosis in primary cultures of rat cerebral cortical and cerebellar granule cells. Neurotox Res 7:319-22.

Tonner PH, Wood SC and Miller KW (1992) Can nicotine self-inhibition account for its low efficacy at the nicotinic acetylcholine receptor from Torpedo? *Mol Pharmacol* **42**:890-897.

Tritto T, McCallum SE, Waddle SA, Hutton SR, Paylor R, Collins AC and Marks MJ (2004) Null mutant analysis of responses to nicotine: deletion of beta2 nicotinic acetylcholine receptor subunit but not alpha7 subunit reduces sensitivity to nicotine-induced locomotor depression and hypothermia. *Nicotine Tob Res* **6**:145-158.

Tsui, AD. Lê, A. Wang, W. Juzytsch and Harding, S (2001) Exposure to nicotine but not alcohol during adolescence enhances acquisition of alcohol consumption in adulthood. Alcohol Clin Exp Res p.25 Suppl: #73 .

Turner JR and Kellar KJ (2005) Nicotinic cholinergic receptors in the rat cerebellum: multiple heteromeric subtypes. *J Neurosci* **25**:9258-9265.

Uzbay IT and Erden BF (2003) Attenuation of ethanol withdrawal signs by high doses of L-arginine in rats. *Alcohol Alcohol* **38**:213-218.

Vernier JM, El-Abdellaoui H, Holsenback H, Cosford ND, Bleicher L, Barker G, Bontempi B, Chavez-Noriega L, Menzaghi F, Rao TS, Reid R, Sacaan AI, Suto C, Washburn M, Lloyd GK and McDonald IA (1999) 4-[[2-(1-Methyl-2-pyrrolidinyl)ethyl]thio]phenol hydrochloride (SIB-1553A): a novel cognitive enhancer with selectivity for neuronal nicotinic acetylcholine receptors. *J Med Chem* **42**:1684-1686.

Vibat CR, Lasalde JA, McNamee MG and Ochoa EL (1995) Differential desensitization properties of rat neuronal nicotinic acetylcholine receptor subunit combinations expressed in Xenopus laevis oocytes. *Cell Mol Neurobiol* **15**:411-425.

Vinceti M, Rothman KJ, Bergomi M, Borciani N, Serra L and Vivoli G (1998) Excess melanoma incidence in a cohort exposed to high levels of environmental selenium. *Cancer Epidemiol Biomarkers Prev* **7**:853-856.

Voogd J and Glickstein M (1998) The anatomy of the cerebellum. *Trends Neurosci* **21**:370-375.

Wada E, Wada K, Boulter J, Deneris E, Heinemann S, Patrick J and Swanson LW (1989) Distribution of alpha 2, alpha 3, alpha 4, and beta 2 neuronal nicotinic receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat. *J Comp Neurol* **284**:314-335.

Wafford KA, Burnett DM, Leidenheimer NJ, Burt DR, Wang JB, Kofuji P, Dunwiddie TV, Harris RA and Sikela JM (1991) Ethanol sensitivity of the GABAA receptor expressed in Xenopus oocytes requires 8 amino acids contained in the gamma 2L subunit. *Neuron* **7**:27-33.

Wang JH and Sun GY (2001) Ethanol inhibits cytokine-induced iNOS and sPLA2 in immortalized astrocytes: evidence for posttranscriptional site of ethanol action. *J Biomed Sci* **8**:126-133.

Wang N, Orr-Urtreger A, Chapman J, Rabinowitz R and Korczyn AD (2003) Deficiency of nicotinic acetylcholine receptor beta 4 subunit causes autonomic cardiac and intestinal dysfunction. *Mol Pharmacol* **63**:574-580.

Wang N, Orr-Urtreger A, Chapman J, Rabinowitz R, Nachman R and Korczyn AD (2002) Autonomic function in mice lacking alpha5 neuronal nicotinic acetylcholine receptor subunit. *J Physiol* **542**:347-354.

Wang ZZ, Hardy SF and Hall ZW (1996) Assembly of the nicotinic acetylcholine receptor. The first transmembrane domains of truncated alpha and delta subunits are required for heterodimer formation in vivo. *J Biol Chem* **271**:27575-27584.

Ward JM, Cockcroft VB, Lunt GG, Smillie FS and Wonnacott S (1990) Methyllycaconitine: a selective probe for neuronal alpha-bungarotoxin binding sites. *FEBS Lett* **270**:45-48.

Ward RJ, Lallemand F, de Witte P and Dexter DT (2008) Neurochemical pathways involved in the protective effects of nicotine and ethanol in preventing the development of Parkinson's disease: potential targets for the development of new therapeutic agents. *Prog Neurobiol* **85**:135-147.

Watson WP and Little HJ (1999) Prolonged effects of chronic ethanol treatment on responses to repeated nicotine administration: interactions with environmental cues. *Neuropharmacology* **38**:587-595.

Wazlawik E and Morato GS (2003) Influence of drugs acting on nitric oxide-dependent pathways on ethanol tolerance in rats. *Psychopharmacology (Berl)* **170**:343-350.

Winzer-Serhan UH and Leslie FM (1997) Codistribution of nicotinic acetylcholine receptor subunit alpha3 and beta4 mRNAs during rat brain development. *J Comp Neurol* **386**:540-554.

Womack MD, Hoang C and Khodakhah K (2009) Large conductance calcium-activated potassium channels affect both spontaneous firing and intracellular calcium concentration in cerebellar Purkinje neurons. *Neuroscience* **162**:989-1000.

Wright JM, Peoples RW and Weight FF (1996) Single-channel and whole-cell analysis of ethanol inhibition of NMDA-activated currents in cultured mouse cortical and hippocampal neurons. *Brain Res* **738**:249-256.

Wu G, Tonner PH and Miller KW (1994) Ethanol stabilizes the open channel state of the Torpedo nicotinic acetylcholine receptor. *Mol Pharmacol* **45**:102-108.

Wùllner U, Gundisch D, Herzog H, Minnerop M, Joe A, Warnecke M, Jessen F, Schutz C, Reinhardt M, Eschner W, Klockgether T and Schmaljohann J (2008) Smoking upregulates alpha4beta2* nicotinic acetylcholine receptors in the human brain. *Neurosci Lett* **430**:34-37.

Wultsch T, Chourbaji S, Fritzen S, Kittel S, Grunblatt E, Gerlach M, Gutknecht L, Chizat F, Golfier G, Schmitt A, Gass P, Lesch KP and Reif A (2007) Behavioural and expressional phenotyping of nitric oxide synthase-I knockdown animals. *J Neural Transm Suppl*:69-85.

Xiao Z, Lu Z, Liu Z, Liu W, Li L, Yin S, Yu S, Dong H and Zhu F (2008) Ethanol inhibits voltage-gated sodium channels in cultured superior cervical ganglion neurons. *Neuroreport* **19**:1773-1776.

Xu W, Orr-Urtreger A, Nigro F, Gelber S, Sutcliffe CB, Armstrong D, Patrick JW, Role LW, Beaudet AL and De Biasi M (1999) Multiorgan autonomic dysfunction in mice lacking the beta2 and the beta4 subunits of neuronal nicotinic acetylcholine receptors. *J Neurosci* **19**:9298-9305.

Yang X, Criswell HE and Breese GR (1999) Action of ethanol on responses to nicotine from cerebellar interneurons and medial septal neurons: relationship to methyllycaconitine inhibition of nicotine responses. *Alcohol Clin Exp Res* **23**:983-990.

Yazdi HH, Janahmadi M and Behzadi G (2007) The role of small-conductance Ca2+-activated K+ channels in the modulation of 4-aminopyridine-induced burst firing in rat cerebellar Purkinje cells. *Brain Res* **1156**:59-66.

Yoshida K, Engel J and Liljequist S (1982) The effect of chronic ethanol administration of high affinity 3H-nicotinic binding in rat brain. *Naunyn Schmiedebergs Arch Pharmacol* **321**:74-76.

Yu CR and Role LW (1998) Functional contribution of the alpha7 subunit to multiple subtypes of nicotinic receptors in embryonic chick sympathetic neurones. *J Physiol* **509 (Pt 3)**:651-665.

Zhang S, Grover GJ, Stokes H, Scholz PM and Weiss HR (2005) Negative inotropic effect of low-dose ethanol in mouse ventricular myocytes is mediated by activation of endothelial nitric oxide synthase. *J Cardiovasc Pharmacol* **46**:343-347.

Zhang W, Edvinsson L and Lee TJ (1998a) Mechanism of nicotine-induced relaxation in the porcine basilar artery. *J Pharmacol Exp Ther* **284**:790-797.

Zhang X, Gong ZH and Nordberg A (1994) Effects of chronic treatment with (+)- and (-)-nicotine on nicotinic acetylcholine receptors and N-methyl-D-aspartate receptors in rat brain. *Brain Res* **644**:32-39.

Zhang X, Liu C, Miao H, Gong ZH and Nordberg A (1998b) Postnatal changes of nicotinic acetylcholine receptor alpha 2, alpha 3, alpha 4, alpha 7 and beta 2 subunits genes expression in rat brain. *Int J Dev Neurosci* **16**:507-518.

Zhang X, Paterson D, James R, Gong ZH, Liu C, Rosecrans J and Nordberg A (2000) Rats exhibiting acute behavioural tolerance to nicotine have more [125I]alpha-bungarotoxin binding sites in brain than rats not exhibiting tolerance. *Behav Brain Res* **113**:105-115.

Zhao R, Chen H and Sharp BM (2007) Nicotine-induced norepinephrine release in hypothalamic paraventricular nucleus and amygdala is mediated by N-methyl-D-aspartate receptors and nitric oxide in the nucleus tractus solitarius. *J Pharmacol Exp Ther* **320**:837-844.

Zima T, Druga R and Stipek S (1998) The influence of chronic moderate ethanol administration on NADPH-diaphorase (nitric oxide synthase) activity in rat brain. *Alcohol Alcohol* **33**:341-346.

Zoli M, Le Novere N, Hill JA, Jr. and Changeux JP (1995) Developmental regulation of nicotinic ACh receptor subunit mRNAs in the rat central and peripheral nervous systems. *J Neurosci* **15**:1912-1939.

Zoli M, Lena C, Picciotto MR and Changeux JP (1998) Identification of four classes of brain nicotinic receptors using beta2 mutant mice. *J Neurosci* **18**:4461-4472.

Zuo Y, Kuryatov A, Lindstrom JM, Yeh JZ and Narahashi T (2002) Alcohol modulation of neuronal nicotinic acetylcholine receptors is alpha subunit dependent. *Alcohol Clin Exp Res* **26**:779-784.

APPENDIX A: ANIMAL USE APPROVAL LETTER

Animal Care and Use Committee
East Carolina University
212 Ed Warren Life Sciences Building
Greenville, NC 27834
252-744-2436 office • 252-744-2355 fax

November 13, 2007

M. Saeed Dar, Ph.D.
Department of Pharmacology
Brody 6S-10
ECU Brody School of Medicine

Dear Dr. Dar:

Your Animal Use Protocol entitled, "Neurochemical/Biochemical Basis of Acute and Chronic Ethanol- and Cannabinoid-Induced Motor Disturbances in Mice," (AUP #W125d) was reviewed by this institution's Animal Care and Use Committee on 11/13/07. The following action was taken by the Committee:

"Approved as submitted"

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

APPENDIX B: BUFFERS, REAGENTS, AND SOLUTIONS

Artificial cerebrospinal fluid (aCSF)

NaOH	27.65mM
KCl	2.55mM
$CaCl_2$	0.05mM
$MgCl_2$	0.94mM
$Na_2S_2O_5$	0.05mM

pH 7.4 (adjusted with NaOH)

Nitrite analysis buffer solution

20mM Tris-HCL buffer and 10mM EDTA (pH 7.4), 100ml

Weigh 242.28mg Tris (molecular weight 121.14)

Add 5 ml of 500 mM EDTA

Add 75 ml distilled H_2O (80 ml total volume)

Add concentrated HCl slowly to pH 7.4 (~4ml)

Add distilled H_2O to volume of 100ml

Nitrite analysis buffer reagents

30mU Nitrate reductase, 100ul sample solution:

Stock 1: add 200ul distilled H_2O to a 10 U vial

Stock 2: add 970 µl distilled water to 30 µl stock 1

Use 20 µl stock 2 for each sample

3uM (molecular weight: 833.4) NADPH, 0.25 µg/100ul sample solution:

Stock 1: weigh 50 µg NADPH, then dissolve in 1000 µl distilled H_2O

Stock 2: add 750 µl distilled H2O to 250 µl stock 1

Use 20 µl stock 2 for each sample

750 µM Glucose-6-phosphate (G-6-P; molecular weight: 340.1),

100 µl sample solution:

Weigh 1.275 mg G-6-P

Add 1000 µl distilled H_2O

Use 20 µl for each sample

48 mU Glucose-6-phosphate dehydrogenase (G-6-PDH), 100 µl sample solution:

Stock 1: add 1000 µl distilled H_2O) to a 100 U vial

Stock 2: add 976 µl distilled H2O to 24 µl stock 1

Use 20 µl stock 2 for each sample

2, 3-Diaminoapthalene (DAN) 50ug/1000ul:

Weigh 50 µg DAN

Add 1000 µl 0.62 N HCl

Add 30 µl to each 100 µl sample solution