Differential Molecular and Genetic Short-term and Long-term Effects of Chronic Nicotine Exposure on Adolescent and Adult Rats

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Dedication

This dissertation is dedicated to my mother Gertrude and my sister Samla for their tireless support throughout this process, and for inspiring me to chase whatever my dreams may be. I could not have made it without you.

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I would like to thank Dr. David Perry, my doctoral advisor, for his guidance and patience during this long process. Thank you for taking a chance on me. You have repeatedly gone above and beyond to help me. I am honored to have been your student. I would also like to thank my dissertation committee Dr. Vincent A. Chiappinelli, Dr. Norman H. Lee, Dr. David S. Mendelowitz, and Dr. Kenneth J. Kellar for all their invaluable guidance and assistance in crafting this dissertation. A special thanks to Dr. Linda L. Werling for the academic and emotional support she's given me. Thanks also to Dr. Jonathan Katz for all you taught me about animal behavioral studies. Finally, thank you to the entire Institute for Biomedical Sciences. It has been a pleasure working along side all of you.

Abstract

Differential Molecular and Genetic Short-term and Long-term Effects of Chronic Nicotine Exposure on Adolescent and Adult Rats

Adolescence is a time of significant brain development, and exposure to nicotine during this period is associated with higher subsequent rates of dependence. Chronic nicotine exposure alters expression of nicotinic acetylcholine receptors (nAChRs), changing the pattern of nicotine responsiveness. I used quantitative autoradiography to measure three major subtypes of nAChRs after chronic nicotine exposure by osmotic minipump in adult (PN60-70) and periadolescent (PN28-30) rats. Comparison of control animals at the two different ages revealed that periadolescents express consistently greater numbers of α4β2* nAChRs compared to the same brain regions of adults. Similar but less pronounced increases in α7 nAChRs were found in control periadolescent rats compared to adults. Binding of [^{125}I]α-conotoxin MII (largely to α6* nAChRs) did not systematically differ between adults and periadolescents. The response to chronic nicotine exposure also differed by age. Up-regulation of α4β2* nAChRs was prominent and widespread in adult animals; in periadolescents, α4β2* up-regulation also occurred, but in fewer regions and to a lesser extent. A similar pattern of response was seen with α7 receptors: adults were more responsive than periadolescents to nicotine-induced up-regulation. In adult animals, chronic nicotine

exposure did not cause up-regulation of α6* nAChRs; binding was down-regulated in three regions. Unlike the other subtypes, the response of α6* nAChRs to chronic nicotine was greater in periadolescents, with more regions showing greater down-regulation compared to adults. These differences in receptor expression and regulation between age groups are likely to be important given the unique vulnerability of adolescents to nicotine-induced behavioral changes and susceptibility to drug abuse.

I used whole genome microarray analysis to determine the effects of nicotine on gene expression in the ventral tegmental area. I examined brains immediately after two weeks of nicotine or saline, and also four weeks after termination of nicotine exposure. After identifying genes with a significant age x treatment interaction, I employed template matching to find specific patterns of expression across age and treatment. Of those genes that were transiently regulated (up- or down-regulated immediately following the end of nicotine treatment, but back to saline baseline 30 days later), two-thirds were specific to adult animals, while only 30% were specific to adolescents and 4% were shared across the two ages. In contrast, significant genes that were persistently regulated (altered following nicotine treatment and still altered 30 days later) were more likely (59%) to be adolescent associated, with only 32% in adults and 8% shared. The greatest number of significant genes was late-regulated (no change immediately after nicotine, but regulated 30 days later). Again, most were in adolescents (54%), compared to adults (10%) or shared (36%). Pathway analysis revealed that adolescent-specific genes were over-represented in several biological functions and canonical pathways, including nervous system development and function

and long-term potentiation. Furthermore, adolescent-specific genes formed extensive interaction networks, unlike those specific for adults or shared between the two age groups. This age-specific expression pattern may relate to the heightened vulnerability of adolescents to the effects of addictive drugs. In particular, the propensity of adolescents to show persistent alterations in gene expression corresponds to the persistence of drug dependence among smokers who began their habit as adolescents. These findings support a model whereby adolescent brains are uniquely vulnerable to long-term changes in gene expression in the brain's reward pathway caused by early exposure to nicotine.

Table of Contents

Dedication iv

Acknowledments v

Abstract vi

Table of Contents ix

List of Figures xii

List of Tables xiv

Abbreviations xv

Introduction 1

1. Increased drug vulnerability during adolescence.....1

2. The developing adolescent brain.....2

3. Defining adolescence in the rat.....2

4. Neurochemical effects of nicotine on adolescent brain.....3

5. Circuitry of the mesolimbic reward pathway.....4

6. Nicotine effects in the meolimbic reward pathway.....5

7. Greater relative behavioral response to nicotine during adolescence.....6

8. Enhanced short-term rewarding effects of nicotine during adolescence.....8

9. Reduced short-term aversive effects of nicotine during adolescence.....9

10. Reduced symptoms of nicotine withdrawal in adolescent animals.....9

11. Enhanced long-term rewarding effects of nicotine in adolescent rodents.....11

12. Adolescent nicotine exposure and neurological disease.....11

13. Nicotine effects on gene expression.....14

14. Hypothesis and specific aims……18

15. Experimental design……19

Methods……22

1. Adolescent and adult nicotine exposure……22

2. Autoradiography……24

3. Tissue preparation, total RNA isolation, and gene expression profiling……27

4. Statistical and pathway analysis……28

5. Quantitative real-time reverse transcriptase PCR……29

Results……31

1. Blood and brain nicotine and cotinine concentrations in adolescent and adult animals……31

2. Radioligand binding to three major nAChR subtypes……34

3. α4β2* receptor numbers in saline treated animals……39

4. α4β2*receptor numbers in nicotine treated animals……45

5. α7 binding in saline treated animals……50

6. α7 binding in nicotine treated animals……50

7. α6 binding in saline treated animals……54

8. α6 binding in nicotine treated animals……54

9. Age-specific gene regulation in the VTA by nicotine……58

10. Shared genes regulated by nicotine……59

11. Age-specific biological functions overrepresented in VTA of nicotine treated animals……63

12. Age-specific canonical pathways in VTA of nicotine treated animals……..68

13. Shared biological functions and canonical pathways overrepresented in nicotine treated animals……71

14. Nicotine regulated genes validated by qRT-PCR……71

Discussion……74

1. Developmental differences in nAChR subtype distribution……74

2. Developmental differences in nAChR function……75

3. α4β2* nAChRs……76

4. α7 nAChRs……76

5. α6* nAChRs……77

6. Age differences in response to chronic nicotine……78

7. Nicotine produces age-specific patterns of gene regulation……80

8. Over-represented biological functions……82

9. Nervous system development and function……83

10. Circadian rhythms……84

11. Long term potentiation (LTP)……85

References……89

List of Figures

Figure 1. Statistical analysis of whole-genome microarray data of adolescent and adult nicotine-treated and saline-treated SD rats……21

Figure 2. Plasma and Brain Nicotine and Cotinine in Brains of Adolescent and Adult Rats after 2 week Nicotine Treatment……33

Figure 3. [^{125}I]A-85380 binding to α4β2* nAChRs in rat brain sections from animals representing the four treatment groups……36

Figure 4. [125I]αBtx binding to α7 nAChRs in rat brain sections from animals representing the four experimental groups……..……..37

Figure 5. [125I]αCtxMII binding to α6* nAChRs in rat brain sections from animals representing the four experimental groups……..……..38

Figure 6. Binding in Control Adolescent Brains as a % of that in Adult Brains……..…43

Figure 7. Binding in Nicotine Treated Adult Brains as a % of Saline Controls……...46

Figure 8. Binding in Nicotine Treated Adolescent Brains as a % of Saline Controls…...48

Figure 9. Gene Expression Correlation with Chronic Nicotine Exposure…….60

Figure 10. Venn Diagram of Template-matched Genes Significantly Regulated by Chronic Nicotine Treatment in Adult and Adolescent Rats……..……62

Figure 11. Over-represented Biological Functions with Possible biological relevance to Chronic Nicotine Treatment……..65

Figure 12. Interaction Network of Genes Significant for Regulation by Chronic Nicotine Falling within Nervous System Development and Function…….67

Figure 13. Canonical Pathways Regulated by Chronic Nicotine……..69

Figure 14. Synaptic Long Term Potentiation Canonical Pathway in Adolescent Rats Treated with Chronic Nicotine……...70

Figure 15. Quantitative Real Time RT-PCR Validation of Microarray Data……...……73

List of Tables

Table 1. List of Primers used for Real-time RTPCR Validation……30

Table 2. Binding (fmoles) of [^{125}I]A-85380 in the presence of 100 nM αCtxMII (to α4β2* nAChRs)……40

Table 3. Binding (fmoles) of [^{125}I]α-bungarotoxin (to α7 nAChRs)……51

Table 4. Binding (fmoles) of [^{125}I]α-conotoxin MII (to α6/α3* nAChRs)……56

Table 5. Numbers and functions of genes identified by Ingenuity Pathways Analysis involved in nervous system development and function in adolescent and adult animals including age-specific and shared genes……66

Abbreviations

[125I]-5-iodo-3-(2(S)-azetidinylmethoxy)pyridine: [125I]A-85380

[125I]α-conotoxin MII: [125I]α-CtxMII

[125I]α-bungarotoxin: [125I]αBtx

AMPA: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

CPA: conditioned place aversion

CPP: conditioned place preference

DA: dopamine

FDR: false discovery rate

GABA: gamma amino butyric acid

GC: gas chromatography

GCOS: Gene Chip Operating Software

IPA: Ingenuity Pathways Analysis

LDTg: laterodorsal tegmental nucleus

LTD: long-term depression

LTP: long-term potentiation

MBH: medial-basal hypothalamus

NAcc: nucleus accumbens

nAChR: nicotinic acetylcholine receptor

NMDA: N-methyl D-asparic acid

PFC: prefrontal cortex

PN: post-natal day

qRT-PCR: quantitative real-time RT-PCR

SD: Sprague-Dawley

VTA: ventral tegmental area

Introduction

1. Increased drug vulnerability during adolescence

Adolescence is the most common period for initiation of recreational drug use (Spear, 2004). Such use often begins with tobacco products, and recent evidence suggests that adolescents are particularly susceptible to the addictive and adverse effects of tobacco smoke. Adolescent use of tobacco is associated with subsequent higher daily consumption and a lower probability of smoking cessation (Chen & Millar, 1998). Nicotine has been shown to have a number of adverse effects on the brain, particularly during early development. Epidemiological studies have demonstrated that adolescent smokers proceed to dependence quicker, are more likely to be dependent as adults, and exhibit increased co-morbidity with other drug use and more psychological problems compared to adults (Chen & Millar, 1998; Chambers et al., 2003; Adriani et al., 2006).

Behavioral studies have shown distinct responses to nicotine in animals treated during adolescence (PN28-42) relative to their adult counterparts. Pre-exposure to nicotine during, but not following, adolescence sensitizes rats to nicotine's effects on locomotor response and conditioned place preference (CPP), and increases self-administration of nicotine (Adriani et al., 2006). Pre-exposure to nicotine during adolescence also sensitizes rats to the rewarding effects of other drugs (Chambers et al., 2003; Adriani et al., 2006), particularly psychostimulants such as cocaine (Collins & Izenwasser, 2004; McMillen et al., 2005; McQuown et al., 2007). These data strongly suggest an increased vulnerability to the effects of nicotine during adolescence.

2. The developing adolescent brain

Adolescence is characterized by extensive physiological and psychological changes. Until recently, the human brain was thought to be fully developed by early adolescence. However, studies have shown that the human brain continues to mature into the early 20's (Sowell et al., 2003). This is characterized by extensive growth during early adolescence, followed by a decrease in grey matter, a gradual loss of synapses, and a strengthening of the remaining synapses. Neurotransmitter receptor populations peak during adolescence and decline thereafter. This has been shown for GABA, serotonin, norepinephrine, dopamine (DA), and acetylcholine receptors (Lujan et al., 2005; Azam et al., 2007). These changes occur throughout the brain and coincide with changes in complex social behaviors characterized by increased impulsivity, risk-taking, and sensation-seeking (Sowell et al., 2003). Exposure to nicotine during this period may have systemic affects that persist into adulthood and contribute to the observed increase in consumption of tobacco and other drugs of abuse, and the subsequent difficulty of quitting in those individuals that began smoking during adolescence.

3. Defining adolescence in the rat

In rats, periadolescence has classically been defined as the time between the earliest detection of diurnal gonadotropin cycling (approximately postnatal day 28 [PN28]) and reproductive maturity (approximately PN38–42) (Spear & Brake, 1983). Neurochemical, neuroanatomical and behavioral changes that occur during this period

in rats are similar to those seen in human adolescents (Spear & Brake, 1983; Slotkin, 2002; Adriani et al., 2003).

4. Neurochemical effects of nicotine on adolescent brain

Nicotine is the major neuroactive and addictive component of tobacco smoke. Nicotine acts on pathways affecting neuronal development and behavior, modulation of anxiety, behavioral inhibition, reward, and habit formation. Nicotine has been demonstrated to cause long-lasting adverse effects on the adolescent rat brain, including altered proliferation, differentiation, synaptic activity, synaptic maturation, and increased cell damage and cell death (Trauth et al., 2000a; Slotkin, 2002; Abreu-Villaca et al., 2003; Abreu-Villaca et al., 2005).

Chronic exposure to nicotine, as well as smoking in humans, is well-known to alter numbers of nicotinic cholinergic receptors (nAChRs) in brain, usually causing up-regulation (Marks et al., 1983; Schwartz & Kellar, 1983; Benwell et al., 1988; Peng et al., 1997; whiteaker et al., 1998). Receptor regulation may contribute to behavioral effects of long-term nicotine exposure, including tolerance and dependence. Although extensive work has detailed the effects of chronic nicotine exposure on different subtypes of nAChRs throughout the adult rat brain, there have been relatively few studies of the effects of such exposure on nAChRs in adolescent rat brain. Animal studies of adolescent nicotine exposure mimicking plasma levels of smokers have demonstrated up-regulation of nAChRs in those brain regions associated with the reward pathway (Jacobs et al., 2002; Slotkin, 2002), and long-term decrements in neural

activity after nicotine withdrawal (Trauth et al., 2000; Trauth et al., 2001; Xu et al., 2001; Slotkin, 2002; Xu et al., 2002). While nicotine can function in a neuroprotective manner in adults, it appears to function as a neurotoxin in fetal and adolescent brains. These findings suggest that adolescence is a critical period during which nicotine use produces distinct effects, possibly contributing to increased susceptibility to addiction in adulthood.

5. Circuitry of the mesolimbic reward pathway

The behavioral effects of nicotine are in part regulated by dopamine transmission in the mesolimbic pathway (Balfour el al., 2002, Corrigall et al., 1992, Mansvelder & McGehe, 2002). The mesolimbic dopamine pathway projects from the cell bodies in the ventral tegmental area (VTA) and terminates in the nucleus accumbens (NAcc), prefrontal cortex and other forebrain regions. The NAcc has been shown to play a role in drug addiction (Koob & Kreck, 2007). DA release into the NAcc is regulated by excitatory and inhibitory projections from the VTA. Glutamatergic inputs from the prefrontal cortex provide the primary excitatory control of VTA neuronal activity (Johnson et al., 1992; Kalivas et al., 1989; Mansvelder et al., 2003; Sesack & Pikel, 1992; Taber & Fibiger, 1995). There is also cholinergic control of VTA DA neurons from the laterodorsal tegmental nucleus (LDTg) (Blaha et al., 1996). Blockade of VTA glutamate receptors inhibits nicotine-induced increases in DA release into the NAcc (Schilstrom et al., 1998) suggesting that nicotine modulates glutamate transmission which regulates VTA dopamine release. Along these lines, Erhardt and colleagues (2002) showed that

systemic nicotine administration produces increased firing in VTA neurons which was inhibited by blockade of local glutamate receptors.

The primary inhibitory inputs to the VTA are GABAergic inputs from local interneurons as well as projections from the NAcc and ventral pallidum. Local blockade of VTA GABA receptors facilitated nicotine-induced dopamine release (Erhadt et al., 2002); suggesting the mesolimbic pathway is under inhibitory control via local GABA transmission in the VTA.

6. Nicotine effects in the mesolimbic reward pathway

nAChRs are expressed in three cell types in the VTA. Dopamine neurons express α2-10 and β2-4 transcripts (Mansvelder et al., 2003). Glutamate terminals in the VTA express α7 receptors which regulate glutamate release. GABA cell bodies express α4β2* receptors which regulate GABA release (Marchi et al., 2002; Nomikos et al., 2000).

Cholinergic drive to the VTA neurons is inhibited by nAChR blockade or desensitization from nicotine exposure. α4β2* receptors on GABA neurons desensitize quickly and recover slowly (Wang & Sun, 2005). Nicotine exposure causes VTA neurons to receive less inhibition from GABA, resulting in increased firing of dopamine neurons (Mansvelder et al., 2002). α7 receptors are less sensitive to nicotine-induced desensitization (Wooltorton et al., 2003). β2-containing nAChRs desensitize rapidly following nicotine exposure, but α7 receptors remain active under similar conditions of nicotine exposure (Dani et al., 2001). Thus, nicotine causes a net excitation of VTA

dopamine neurons resulting in enhanced dopamine release in the NAcc. These changes in the VTA persist well after withdrawal signs have disappeared (Pulvirenti & Diana., 2001) suggesting long-term changes in plasticity. Nicotine infusion into the VTA produces long-term increases in NAcc dopamine release (Nisell et al., 1994).

Nicotine has been shown to produce greater increases in dopamine and serotonin release in adolescent rodents compared to adult (Shearman et al., 2008). Nicotine increases [3H]dopamine release from synaptosomes of adolescent rats compared to adult rats (Azam et al., 2007). This increase is only observed in male rats, likely due to sex differences in the maturation of dopamine systems rather than acute differences in sex hormones.

7. Greater relative behavioral response to nicotine during adolescence

Adolescent rats differ from adult rats in their nicotine-induced behavioral responses (Belluzzi et al., 2004; Adriani et al., 2003), and the behavioral effects of adolescent nicotine exposure can persist into adulthood. Nicotine has been shown to produce conditioned place preference (CPP) in adolescent male Sprague-Dawley rats, but not in their adult counterparts, and multiple exposures to nicotine produces stimulatory effects in adolescent rats not seen in adult rats (Vastola et al., 2002), suggesting greater relative sensitivity. Pre-exposure to nicotine during, but not following, adolescence sensitizes rats to nicotine effects on CPP and locomotion during adulthood (Adriani et al., 2006). Also, several recent studies (although not all; (Kelley & Middaugh, 1999)), have shown that pretreatment with nicotine during adolescence

sensitizes rats to the rewarding effects of other drugs (Collins & Izenwasser, 2004; McMillen et al., 2005; McQuown et al., 2007).

Adolescent drug use could potentially alter brain development and ultimately affect normal adult behavior. Nicotine has been demonstrated to act on pathways affecting neuronal development and behavior. Nicotine acts on mesolimbic and nigro-striatal neurons to modulate anxiety, behavioral inhibition, reward, and habit-forming pathways (Picciotto, 1998). The rewarding effects of psychostimulant drugs are ascribed to the mesolimbic dopaminergic projections (Robbins & Everitt, 2002). The mesolimbic system plays a major role in nicotine-induced locomotion and sensitization as evidenced by the production of locomotor stimulation in response to targeted injection of nicotine into the VTA (Reavill & Stolerman, 1990). This response was abolished by lesion of the nucleus accumbens. Nicotine has also been demonstrated to induce CPP (Calcagnetti & Schechter, 1994; Vastola et al., 2002; Belluzzi et al., 2004; Le Foll & Goldberg, 2005), produce sensitization as measured by increased locomotor activity (Bevins & Palmatier, 2003; Belluzzi et al., 2004; Schochet et al., 2004; Tapper et al., 2004; Cruz et al., 2005), and is self-administered by a number of species (Corrigall et al., 1992; Suto et al., 2001; Levin et al., 2003; Paterson & Markou, 2004; Rahman et al., 2004; Belluzzi et al., 2005).

The effects of drug use during this period of cortical brain changes are only beginning to be examined. Therefore, it is vital to examine the structural, neurochemical, and genetic influences of drug use on the neurodevelopment of the adolescent brain, and its subsequent effects on adult neurobiology and behavior, if we

are to answer critical questions about the effects of adolescent drug use. Specifically, how does adolescent drug use alter normal neurodevelopment? Which alterations persist into adulthood, and how does this affect addictive behavior during adulthood?

8. Enhanced short-term rewarding effects of nicotine during adolescence

Drugs produce long-term and short-term effects which influence future use of that drug and possibly other drugs. Nicotine produces both positive and negative short-term and long-term effects. Recent evidence suggests adolescent animals display enhanced rewarding effects of nicotine as well as reduced aversive effects (O'Dell et al., 2010). Adolescent rats have been shown to acquire nicotine self-administration more quickly and reach higher levels of self-administration relative to their adult counterparts under both limited (Levin et al., 2003, 2007) and extended (Chen et al., 2007) access conditions. Oral self-administration studies show adolescent rats have elevated nicotine intake relative to adult rats. Early adolescent mice have a stronger preference for nicotine in their drinking water, and will compensate for decreased dose by increasing intake (Adriani et al., 2002).

CPP studies, which are used to measure conditioned rewarding effects, have also been used to study the rewarding effects of nicotine. Adolescent animals display enhanced nicotine induced CPP across a wide range of doses. Adolescent animals develop CPP at doses that do not produce CPP in adult animals (Shram et al., 2006, Vastola et al., 2002). Further, Belluzi and colleagues (2004) showed that a single injection of nicotine was sufficient to cause CPP in early adolescents (postnatal day 28;

PN28) but not in late adolescents (PN38) or in adults (PN70) even after four subsequent additional injections. Mouse studies mirror these results (Kota et al., 2007). This suggests enhanced short-term rewarding effects contribute to increased use of nicotine during adolescence.

9. Reduced short-term aversive effects of nicotine during adolescence

Studies show adolescent rodents also experience reduced aversive effects of nicotine relative to adult rodents. High doses of nicotine have been shown to cause conditioned place aversion (CPA) in adult rats, a response that is not observed in their adolescent counterparts (O'Dell 2009). Recent studies further suggest that adolescent rodents experience reduced anxiogenic effects of nicotine relative to adult rodents. Nicotine stimulates the hypothalamic pituitary adrenal axis producing increased plasma levels of corticosteroids in adult rats (Cao et al., 2007). The effect was absent in adolescents. Behavioral studies show that following 12 days of oral nicotine, adolescent mice show reduced anxiety-like behavior measured by elevated plus maze. Other studies suggest nicotine may have anxiolytic effects in adolescent rodents. CPA studies show, in adolescent but not adult rats, nicotine increases the time spent in a previously non-preferred side (Torrella et al., 2004).

10. Reduced symptoms of nicotine withdrawal in adolescent animals

Evidence also suggests adolescent rodents experience reduced nicotine withdrawal symptoms relative to adult rodents. In withdrawal studies, animals are

typically treated with nicotine for 5-7 days, followed by initiation of withdrawal. Withdrawal is initiated either by removal of the nicotine (spontaneous withdrawal) or administration of the non-selective nicotinic antagonist mecamylamine (precipitated withdrawal). The animals are then observed for signs of withdrawal (e.g. abdominal constriction, facial fasciculation, eye blinking, gasps and writhes). Adolescent rats showed fewer signs of withdrawal than adult rats receiving the same dose of nicotine after mecamylamine administration (O'Dell et al., 2007). Differences were significant, however less striking, when withdrawal was spontaneously initiated. Mouse studies show similar results with adolescent mice displaying fewer signs of withdrawal under both spontaneous and precipitated withdrawal conditions (Kota et al., 2007).

Intra cranial self-stimulation measures the affective properties of drugs. In these studies, rats self-administer a small current to a brain region containing efferent projections to reward related structures in the mesolimbic pathway. This has been shown to be strongly reinforcing. Adult nicotine-dependent rats have a higher current intensity threshold during withdrawal (Kenny & Markou, 2001; Paragius et al., 2000). This is a believed to reflect decreased brain reward function. O'Dell (2009) showed that adult nicotine-dependent rats display an increased current intensity threshold under spontaneous and precipitated withdrawal conditions. This effect was not seen in adolescent nicotine-dependent rats. Elevated plus maze experiments show adult rodents experiencing nicotine withdrawal display a decrease in open-arm time (increased anxiety-like behavior) relative to controls. This effect is significantly reduced in adolescent rodents experiencing withdrawal (Wilmouth & Spear, 2006; Kota et al.,

2007). Taken together, these data suggests adolescent rodents experience fewer negative effects during withdrawal relative to adult rodents.

11. Enhanced long-term rewarding effects of nicotine in adolescent rodents

The literature shows that pre-exposure to nicotine during adolescence enhances the rewarding effects of nicotine and other drugs. Adult rats that self-administer nicotine during adolescence self-administer more nicotine than adult rats that initiate nicotine self-administration during adulthood (Levin et al., 2003). Further, adult rats receiving nicotine injections during adolescence self-administer more nicotine than adult rats receiving nicotine injections in adulthood. CPP studies show similar long-term effects. Nicotine pre-exposure during adolescence increases nicotine-induced CPP relative to nicotine pre-exposure during adulthood (Adriani et al., 2006).

12. Adolescent nicotine exposure and neurological disease

An association between smoking tobacco and neurological disease has long been observed in the literature. Among the strongest of these associations is nicotine addiction in schizophrenia. Rates of nicotine addiction in schizophrenics and other psychiatric populations are well above that of the general population. Schizophrenics and other psychiatric populations collectively smoke up to 50% of the cigarettes consumed in the US (Lasser et al., 2000). Further, schizophrenic patients have among the highest rates of smoking (70%-90%) in any population (Buckley, 1998). The majority of the current literature supports the hypothesis that schizophrenics use nicotine as self-

medication. It has been suggested that schizophrenics self-treat with nicotine as a result of a helpful interaction between disease-specific nAChR abnormalities in schizophrenia and nicotine's cognitive and mood-altering properties (Leonard et al., 2001). For instance, nicotine has been shown to improve cognitive and sensory gating deficits seen in schizophrenics (Breese et al., 2000; George et al., 2006). However, this theory seems to clash with our understanding of drug addiction, in that the criteria for addiction is that drug taking becomes compulsive despite negative consequences rather than because of positive consequences. Positive outcomes are neither necessary nor sufficient as a mechanism of drug addiction. There seems little doubt that these are interacting phenomena, however, the nature of this interaction is yet to be clarified.

A growing number of researchers are beginning to suggest the relationship between nicotine consumption and schizophrenia may represent a more general interaction between disease states (Chambers et al., 2001). Substance abuse disorders involving cocaine, alcohol, and cannabis are all increased in schizophrenia well above the general population and with proportional increases similar to smoking (Dixon 1999; Regier et al., 1990). Other studies raise the possibility that nicotine exposure during adolescence may predispose or enhance mental illness in vulnerable populations (Slotkin, 2008).

A growing body of evidence suggests that the neurobiology of schizophrenia may contribute to the observed vulnerability to addiction by facilitating neural substrates that regulate positive reinforcement, incentive motivation, behavioral inhibition, and addictive behavior. While the rate of tobacco use in schizophrenics is significantly

higher than the general population, there is no consensus for substance use co morbidity to be linked to any subset of symptoms (Lambert et al., 1997, Van Ammers et al., 1997). Rather, studies suggest drug availability is the primary determinant of the type of drugs patients use (Baigent et al., 1995; Lambert et al., 1997). Further, standard medication treatments known to be effective for a variety of the symptoms of schizophrenia are not effective in reducing substance abuse in cases of dual diagnosis (Selzer & Lieberman, 1993).

The mesolimbic dopamine system is the major neural substrate for the reinforcing effects of psychostimulants, ethanol, nicotine, and cannabinoids (Fibiger et al., 1992; Wise 1993; Koob et al., 1992a, 1992b). The mesolimbic dopamine system is also implicated in schizophrenia. This is supported by the finding that typical neuroleptics act via blockade of DA receptors and that psychostimulants elevate DA levels in the NAcc, causing psychotic symptoms (Crow et al., 1977; Lieberman et al., 1990). The dopamine hypothesis suggests that schizophrenia symptoms emerge from a functional hyperactivity of DA neurons projecting to the NAcc and hypoactivity of DA neurons projecting to the frontal cortex (Davis et al., 1991; Deutch, 1993; Goldstein & Deutch, 1992). Other studies suggest abnormal glutamatergic cortical activity associated with DA dysregulation in the NAcc may underlie schizophrenic psychosis and thought disorder (Csernansky & Bardgett, 1998) contributing to improper gating of perceptual and thought processes (Hoffman & McGlashan, 1993; Weinberger et al., 1994). NAcc spiny neurons receive excitatory inputs from the prefrontal cortex and hippocampus and DA inputs from the VTA integrating cortical, hippocampal and DA

signals regulating the motivational aspects of drugs and drug related stimuli. The primary neuropathology in schizophrenia remains unknown, but similar dysregulation of the corticolimbic dopamine system may underlie aspects of both schizophrenia and drug addiction. Thus elevated nicotine consumption among schizophrenics could be viewed as a consequence of common disease pathways, rather than an attempt to self-medicate.

13. Nicotine Effects on gene expression

Several studies have demonstrated that gestational nicotine exposure elicits aberrant expression of genes involved in differentiation and apoptosis leading to abnormal cell death, cell arrest, and decreased cell number. Adolescent rats treated with nicotine exhibit up-regulation of p53 (a tumor suppressor gene involved in regulation of differentiation and apoptosis) similar to the gestational nicotine exposure model (Trauth et al., 2000). Corresponding significant decreases in cell numbers were also observed in the cerebral cortex, midbrain, and hippocampus. Though these changes were less pronounced than in the gestational model, the similar trend suggests that the adolescent brain is also vulnerable to the effects of nicotine. Nicotine has also been shown to up regulate delta-fosB (a member of the fosB transcription factor family) in neurons of the NAcc and dorsal striatum (Nestler et al., 2001). fosB persists in neurons for long periods of time, and is believed to contribute to increased susceptibility to the long-term behavioral effects of addictive drugs and increased drug seeking behavior. Two possible mechanisms for the long-term alteration of behavioral

plasticity by fosB and other transcription factors altered by chronic nicotine exposure include the observed modification of neuronal morphology, and permanent modification of gene expression via chromatin remodeling. Neurons from the NAcc of male Long-Evans hooded rats treated with chronic nicotine have been shown to have longer dendrites and an increased number of dendritic segments relative to control animals (McDonald et al., 2005). A subsequent study by the same group demonstrated that these anatomical changes are associated with enduring psychomotor alterations (McDonald et al., 2007). There have been few comprehensive studies of altered neuronal gene expression in a chronic nicotine model to date.

Kane and colleagues (2004) have recently used a pathway-focused microarray approach to examine the effects of chronic nicotine on protein modification/degradation pathways in the prefrontal cortex (PFC) and medial-basal hypothalamus (MBH) of rats chronically treated with nicotine. Chronic nicotine treatment was shown to up-regulate a number of ubiquitin-proteasome and CCT family genes in rat PFC, and down-regulate these genes in the MBH (Kane et al., 2004). The ubiquitin-proteasome pathway is responsible for the majority of intracellular protein degradation and is critical to cell cycle progression, apoptosis, signal transduction, and a number of other vital cellular processes (Conaway et al., 2002). In a separate study, Li and colleagues (2004) examined the effects of chronic nicotine treatment on gene expression in the PFC, amygdala, striatum, medial basal hypothalamus and VTA of male Holtzman rats using a pathway-focused microarray. Their results suggest that nicotine differentially regulates gene expression in a time- and region-specific manner.

Expression data suggest that chronic nicotine exposure leads to global changes in gene expression affecting pathways in brain regions critical to addictive behaviors. However, there has been little examination of these changes in the context of long-term addictive behaviors. These studies were conducted immediately after cessation of nicotine treatment. In order to identify genetic changes contributing to the long-term changes caused by nicotine treatment, expression profiling must be done after a long period of withdrawal. Furthermore, we know of no studies to date examining the effects of chronic nicotine exposure on gene expression in the adolescent brain.

Our general strategy is to identify higher level biological processes involved in addiction over-represented in genes regulated by chronic nicotine exposure in adolescent and adult rats. Recent studies have taken a somewhat similar approach to our own (Sun & Zhao, 2010; Wang & Li, 2010). One major difference however is that these studies begin with a limited set of addiction related candidate genes chosen from the literature, whereas we have taken a whole genome approach. Another significant difference is that there is no age component to these studies. However, one would expect there to be some overlap between nicotine regulated genes in adolescent and adult animals. Further, there is likely to be overlap in the higher level biological systems involved in general addiction. Genome-wide association studies have identified regions on chromosomes 4, 5, 9-11 and 17 as likely to contain susceptibility genes for general addiction (Li & Burmeister, 2009). In a recent meta-study, Li and Burmeister (2009) identified a set of 62 candidate genes for addiction susceptibility based on a stringent literature search. These genes include nAChRs which have been associated with

nicotine, alcohol, and cocaine dependence. For instance, studies have shown an association between the α4 subunit and nicotine dependence (Feng et al., 2004; Li et al., 2005). The β2 subunit has been associated with nicotine dependence (Li et al., 2008) and alcohol dependence (Ehringer et al., 2007). The α3, α5, and β4 subunits have been associated with alcohol dependence (Wang et al., 2008) and cocaine dependence (Grucza et al., 2008).

This list also includes the $GABA_A$ receptor subunit 2 shown to be associated with alcohol dependence (Edenberg et al., 2004; Covault et al., 2004; Lappalainen et al., 2005; Fehr et al., 2006), nicotine dependence (Agrawal et al., 2008) and poly-substance abuse (Drgon et al., 2006); ANKK1, a kinase gene associated with nicotine dependence (Gelernter et al., 2006; Huang et al., 2009) and alcohol dependence (Dick et al., 2007; Yang et al., 2007); and a number of cell adhesion genes including neurexins which have been associated with nicotine dependence (Nussbaum et al., 2008; Bierut et al., 2007), alcohol dependence (Hishimoto et al., 2007), poly-substance abuse (Uhl et al., 2008), and opioid dependence (Lachman et al., 2007). Mutations in the neurexin gene family have also been associated with schizophrenia (Rujescu et al., 2009). We have identified a number of schizophrenia related genes that are regulated in an age specific manner by nicotine. This may suggest overlap in the genes and biological processes involved the etiologies of schizophrenia and addiction.

Sun and Zhao (2010) in a separate study examined this same list of 62 addiction related genes for patterns in higher level biological processes. They identified 44 gene ontology (GO) terms overrepresented in this group of genes, the majority of which were

involved in neural development. Six of the GO terms were related to glutamate and acetylcholine neurotransmission. Thirteen overrepresented canonical pathways were identified including serotonin, glutamate, and dopamine receptor signaling, LTP and LTD, and circadian rhythm signaling.

In a separate meta-study, Wang and Li (2010) identified 16 genes from the literature related to smoking initiation and progression, 99 genes related to nicotine dependence, and 75 genes related to smoking cessation. Bioinformatic analysis of these sets of genes identified 9 pathways overrepresented in the smoking initiation and progression genes, 21 pathways overrepresented in the nicotine dependence genes, and 13 pathways overrepresented in the smoking cessation genes. Four pathways were common to all three lists: calcium signaling, cAMP-mediated signaling, dopamine receptor signaling, and G-protein coupled receptor signaling. Five pathways were shared by 2 of the 3 lists including serotonin receptor signaling, tryptophan metabolism, neurotrophin/TRK signaling, synaptic LTP, and tyrosine metabolism.

14. Hypothesis and Specific Aims

We hypothesize that exposure to chronic nicotine during adolescence has distinct long-lasting behavioral, neurochemical, and genetic effects on the developing adolescent brain which contribute to increased vulnerability to addiction in adulthood. The overarching goal of this dissertation was to examine the short-term and long-term effects of adolescent exposure to nicotine on brain neurochemistry, and on gene expression in the VTA. This dissertation addresses three major questions.

1. How does chronic nicotine exposure during adolescence affect the numbers of the three major subtypes of neuronal nAChRs across brain regions?

2. How does chronic nicotine exposure during adolescence affect gene expression in the VTA?

3. What biological processes and pathways contribute to the increased vulnerability of adolescents to the effects of nicotine?

The three specific aims of this study are:

1. Quantify the expression of the three major nAChR subtypes (α4β2*, α6*, and α7) across a number of brain regions in adolescent and adult nicotine-treated and saline-treated SD rats.
2. Characterize short-term and long-term whole genome changes in expression in the VTA of adolescent and adult nicotine-treated and saline-treated SD rats.
3. Identify shared and age-specific classes of genes (biological processes, gene functions, and canonical pathways) overrepresented in nicotine-treated adolescent and adult SD rats.

15. Experimental Design

In this study, animals received chronic nicotine infusions for 14 days via osmotic minipumps starting in adolescence (PN28-30) or in adulthood (PN60-70). Half of the animals were sacrificed at this point permitting identification of the short-term effects of chronic nicotine in adolescent and adult animals. The remaining animals were

allowed to recover for 30 days and were then sacrificed, permitting identification of the long-term effects of chronic nicotine in adolescent and adult rats. Numbers of nAChRs were determined by radioligand binding and autoradiography. I measured the numbers of all three major nAChRs in numerous brain regions of adolescent and adult nicotine and saline treated animals using [^{125}I]A-85380 to label β2-containing receptors, [^{125}I]αBtx to label α7 homomeric receptors, and [^{125}I] αCtxMII to label α6-containing receptors. I used a whole genome rat array to search a broad range of biological processes and functions significant for regulation by nicotine in three treatment groups: saline, nicotine, and nicotine +30 day recovery. Individual one-way ANOVAs for each age group identified genes with a significant treatment effect. Comparison of these two lists identified genes significant for nicotine regulation in common between adolescents and adults. Functional analysis of these genes reveals the biological functions and pathways in common to rats exposed to chronic nicotine at any age. Two-way ANOVA (treatment x age) was used to identify genes with differential treatment effects with respect to age. Pavlidis template matching was used to identify genes showing three distinct temporal patterns of expression. This design allowed the identification of age-specific and shared nicotine-regulated genes with early, late, and persistent regulation. Functional analysis of these sets of genes was done to identify over-represented biological functions, gene networks, and canonical pathways contributing to the etiology of nicotine addiction specific to each age group.

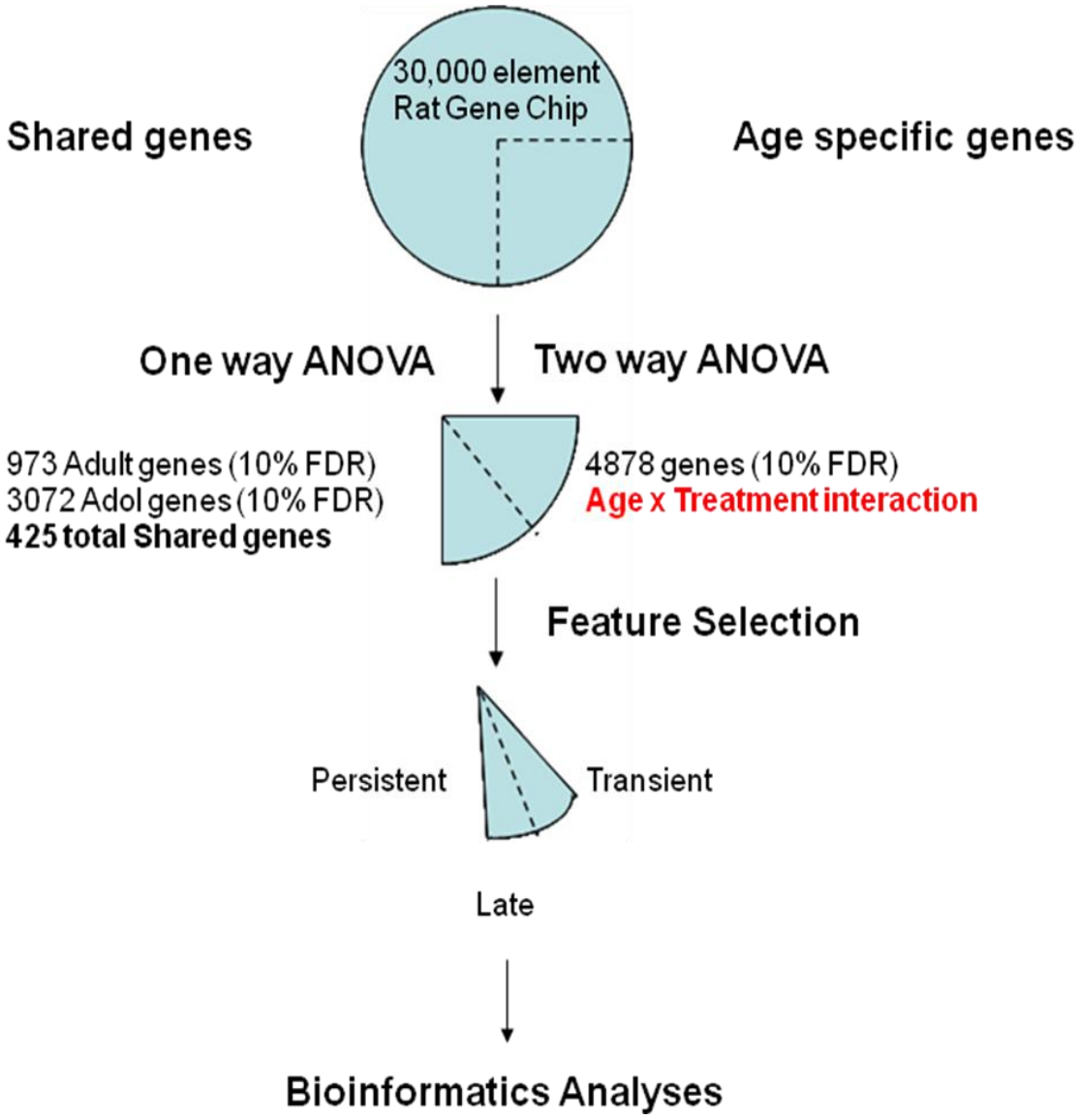

Figure 1. Statistical analysis of whole-genome microarray data of adolescent and adult nicotine-treated and saline-treated SD rats.

Methods

1. Adolescent and adult nicotine exposure

Osmotic minipumps (Alzet model 2002; Durect Corporation, Cupertino, CA) were filled with sterile saline or with nicotine hydrogen tartrate dissolved in saline, at concentrations calculated to achieve a dose of 6 mg/kg/day, calculated as nicotine free base (37 µmol/kg).

Minipumps were implanted into male Sprague–Dawley rats (Hilltop Lab Animals, Scottdale, PA) of two ages, postnatal day (PN) 28-30 or PN 60–70. The period from PN 28–40 in rats is typically labeled periadolescence, that from PN 40–52 middle adolescence, and PN 52–60 late adolescence; "puberty" generally occurs during the last days of periadolescence (Spear, 2004). Thus, our treatment was performed at the early periadolescent and adult stages. Rats were anesthetized with isoflurane and the minipumps inserted into a subcutaneous pocket via a small incision made over the shoulders. While under anesthesia, animals were administered buprenorphine (0.1 mg/kg, s.c.) for post-operative pain. The wound was closed with clips and the area swabbed with antiseptic. After recovery from anesthetic (10–30 min), animals were returned to individual cages. Fourteen days after minipump implantation (PN 42-44 for adolescents PN 74–84 for adults), some of the animals implanted at each age were lightly anesthetized with isoflurane and sacrificed by decapitation. The remaining animals were left untreated for 30 days (minipumps empty 14 to 17 days after implant) during which time the adolescents reach full adulthood. The remaining animals were then sacrificed as described.

For the autoradiography experiment, I used 4 experimental groups: adolescent saline and nicotine, and adult saline and nicotine. These animals were all from the group sacrificed immediately after 2 week treatment. Each experimental group was comprised of 8 animals. Following decapitation, brains were rapidly removed and frozen on dry ice. Frozen coronal brain sections (16 μm) were cut and mounted onto Superfrost Plus slides (Fisher Scientific, Newark, DE) and stored at − 80 °C until use. For each of the three autoradiographic experiments described below, sections from all four treatment groups were incubated together and in random order, to avoid artifacts from in vitro processing.

For the microarray experiment, I used 6 experimental groups, comprised of saline, nicotine, and nicotine + 30 days (nicotine withdrawal), for both the adolescent and adult animals. Each experimental group was comprised of 6 animals. Rats were anesthetized with isoflurane and decapitated; brains were quickly removed and frozen on dry ice. Brain slices were made with a cold stainless steel rat brain slicer matrix (Zivic Instruments, Pittsburgh, PA) with 1 mm coronal slice section intervals. Brain slices were immediately placed in cold saline and four punches (two from each side) were taken from the VTA of each animal using a 500 micron tissue biopsy punch (Zivic Instruments, Pittsburgh, PA). Identification of tissue was done by reference to a rat brain atlas (Paxinos & Watson, 2007); coronal sections were used from -4.6 mm to -5.0 mm from bregma. Tissue punches were stored in RNAlater (Qiagen, Valencia, CA) at -80^{o}C.

Because the per-weight dose changes as animals grow, we used a parallel set of animals to test for the blood and brain levels of nicotine and cotinine occurring in adolescent and adult rats treated with nicotine as described. Animals were weighed prior to sacrifice; trunk blood was collected and plasma prepared and frozen. Forebrains were then extracted as previously described (Ghosheh et al., 2001). Briefly, forebrains were removed, rinsed in saline and dried, then homogenized in 3 volumes of ice-cold 1.15% KCl. After centrifugation for 30 min at 3000 ×g at 4 °C, the supernatant was treated with 1 ml of 2% w/v ZnSO4 for 1 h at 34 °C to precipitate proteins. This mixture was then centrifuged at 30,000 ×g for 60 min at 4 °C, after which the supernatant was removed and frozen.

Concentrations of nicotine and cotinine were determined in the laboratory of Dr. Neal Benowitz (San Francisco General Hospital Clinical Pharmacology Laboratory) by gas chromatography (GC) with nitrogen–phosphorus detection (Jacob et al., 1981), modified for simultaneous extraction of nicotine and cotinine, and determination using capillary GC (Jacob et al., 1991). The internal standards, 5-methylnicotine and ortho-cotinine, were obtained from Dr. Peyton Jacob, III (Division of Clinical Pharmacology of the Department of Medicine, University of California, San Francisco).

Animal use and procedures were approved by the George Washington University Medical Center Institutional Animal Care and Use Committee.

2. Autoradiography

[125I]α-conotoxin MII ([125I]α-CtxMII) was synthesized by the method of Whiteaker et al. (2000), as adapted by us (Perry et al., 2007). Briefly, Tyr0-α-CtxMII (kind gift of J. Michael McIntosh) was reacted with 10 mCi Na125I (22 µl; Perkin-Elmer Life Sciences, Boston, MA) using the chloramine-T method. The reaction mixture was then purified by reversed phase HPLC and fractions collected. This protocol readily separates unreacted Tyr0-α-CtxMII from the mono-iodo and di-iodo forms (Whiteaker et al., 2000). Only the mono-iodo form was utilized, and based on the purification was assumed to be maximally iodinated (2200 Ci/mmol). [125I]A-85380 and [125I]αBtx were purchased from Perkin-Elmer Life Sciences (Shelton, CT). All other chemicals not otherwise mentioned were obtained from Sigma-Aldrich (St. Louis, MO).

[125I]α-CtxMII autoradiography methods were adapted from Perry et al. (2007). Sections were pre-incubated for 15 min in buffer 1 (20 mM HEPES, pH 7.5, 144 mM NaCl, 1.5 mM KCl, 2 mM CaCl2, 1 mM MgSO4, 1 mM PMSF, 0.1% BSA) at room temperature. This was followed by incubation for 60 min at room temperature in buffer 2 (= buffer 1 plus 5 mM EGTA, 5 mM EDTA, 10 µg/ml aprotinin, 10 µg/ml pepstatin A, 10 µg/ml leupeptin) containing 0.8 nM [125I]α-CtxMII (2200 Ci/mmol). Adjacent sections were incubated in the same buffer with 100 µM nicotine added to determine non-specific binding. Slides were then rinsed for 5 min at room temperature in buffer 1, followed by 10 min in buffer 1 on ice, then sequential dips in ice-cold 5 mM HEPES and H2O followed by rapid air-drying.

Autoradiography for [125I]-5-iodo-3-(2(S)-azetidinylmethoxy)pyridine ([125I]A-85380) was adapted from Perry et al. (2007) Sections were first pre-incubated in buffer

1 for 15 min at room temperature, followed by incubation for 60 min at room temperature in buffer 1 with 0.6 nM [125I]A-85380 (2200 Ci/mmol) plus 100 nM unlabeled α-CtxMII. Adjacent sections were incubated in the same buffer with 100 µM nicotine added to determine non-specific binding. After incubation, sections were rinsed twice for 5 min in buffer 1, followed by a water dip and rapid air-drying.

[125I]α-bungarotoxin ([125I]αBtx) autoradiography was adapted from Rasmussen and Perry (2006). Sections were pre-incubated for 30 min at room temperature in 50 mM Tris–HCl, pH 7.3, containing 0.1% BSA, then transferred to this same buffer containing 0.72 nM 125I-α-bungarotoxin (120 Ci/mmol). Adjacent sections were incubated in the same buffer with 100 µM nicotine added to determine non-specific binding. After 120 min incubation at room temperature, sections were then dipped in ice-cold Tris buffer, and then rinsed three times for 10 min in ice-cold buffer, followed by a dip in distilled water and rapid air-drying.

After overnight desiccation, the sections were apposed to film (Kodak BioMax MR) for 4 days (for [125I]α-CtxMII and [125I]A-85380) or 11 days (for [125I]α-bungarotoxin) along with plastic 125I standards (GE Healthcare, Piscataway, NJ); film was developed in an automatic developer. Film images were digitized and quantitative densitometric analysis of binding was done using the Loats Inquiry digital densitometry system (Loats Associates, Winchester, MD). Quantification of binding was done by comparison with standard curves constructed from 125I standards; regions were identified by comparison with the rat brain atlas of Paxinos and Watson (2007). Non-specific binding in adjacent sections was subtracted from the total binding in the paired

section to calculate specific binding. While eight animals were used for each experimental group, for some regions the number of replicates measured per treatment group was less (5–8) due to damaged sections or other technical considerations.

Log-transformed means of specific binding in individual brain regions for each radioligand were compared using two-way ANOVA (SigmaStat 3.5) followed by Holm–Sidak post-hoc comparison of the four treatment groups; differences were accepted at $p < 0.05$. To determine the global effects of age and treatment on binding of each receptor subtype, we used the general linear model (GLM) procedure in SAS (version 8) to test main effects and interactions for the age and treatment variables. Due to non-normality of the dependent variables of the [125I]α-CtxMII binding, even after a log transformation, the GLM procedure was done on the ranks of the variables.

3. Tissue preparation total RNA isolation and gene expression profiling

Each VTA sample (representing an independent biological replicate) consisted of a pool from two animals (a total of 8 punches). Total RNA was isolated and purified using Trizol reagent (InVitrogen, Carlsbad, CA) and the RNeasy micro kit (Qiagen), respectively, according to the manufacturers' instructions. The purified RNA was amplified using the WT-Ovation Pico RNA Amplification System (Nugen, San Carlos, CA) according to the manufacturer's instructions. cRNA target synthesis, hybridization onto the Rat 230 2.0 Gene Chip, and posthybridization staining and scanning were performed using standard protocols as recommended by the manufacturer (Affymetrix, Santa Clara, CA). Chip data were scaled using Gene Chip Operating Software (GCOS) version

1.4 and expression values were log_2-transformed. In total, 18 hybridizations were performed representing 3 independent replicates for each of 3 treatment groups (saline, nicotine, nicotine +30 days) in both adolescent and adult animals.

4. Statistical and pathway analyses

GCOS scaled log_2-transformed expression data were analyzed using the Partek Genomic Suite (Partek, St. Louis, MO). We performed a one-way ANOVA followed by a 10% false discovery rate (FDR) to identify genes with a significant treatment effect in common between both age groups (Shared genes). To identify age-specific genes with a significant treatment effect (Age-specific genes), we performed a two-way ANOVA (age and treatment as the main factors) with 10% FDR and identified genes with a significant age X treatment interaction. The resulting significant genes were subjected to Template Matching/Feature Selection (Pavlidis & Noble, 2001) executed in MEV (www.tm4.org/mev) in order to identify genes exhibiting specified patterns of expression. This search is based on a Pearson Correlation and significance was determined at $P < 0.05$. This design allows me to identify transient, persistent, and late response gene expression profiles that were unique to or shared between the two age groups. Further bioinformatics analysis was conducted on the significant genes to identify functional significance with respect to gene ontology, molecular networks and canonical pathways by means of Ingenuity Pathways Analysis (IPA 6.5 software; Ingenuity Systems, Redwood City, CA) and GeneSpring GX 11 (Agilent Technologies,

Santa Clara, CA). Multigene interaction networks were produced using Pathway Studio 6 (Ariadne Genomics, Rockville, MD).

5. Quantitative real-time reverse transcriptase PCR

To validate expression data for selected genes, we performed quantitative real-time RT-PCR (qRT-PCR) using SYBR green on an ABI Prism 7700 Detection System (Applied Biosystems, Foster City, CA). Quantitative real-time PCR validation was performed on a separate set of animals receiving similar treatment to those used for microarray analysis. Total RNA was reverse transcribed using random primers from TaqMan Reverse Transcription Reagents (Applied Biosystems) per the manufacturer's instructions. PCR primers were chosen for specificity by NCBI BLAST of the rat genome and amplicon specificity was verified by first-derivative melting curve analysis with software provided by Perkin Elmer and Applied Biosystems. Quantitation and normalization of relative gene expression was accomplished using the comparative threshold cycle method previously described (Joe et al., 2005). The house keeping genes GLI1 and RPL36AL exhibited no differential expression in our array analysis and were used for normalization of target genes. Table 1 shows genes identified as differentially expressed across age and treatment groups and chosen for qRT-PCR validation.

Tabel 1. List of primers used for real-time qRT-PCR validation. Shown are the NCBI accession numbers, primer sequences and product sizes.

Gene	Accession #	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon
CTNNB1	NM_053357	TCATGCGCCTTTGCGGGAAC	CGGACGCCCTCCACGAACTG	126
DDR1	NM_001166022	AGCCAAGTGCCGCTATGCCC	GGCACCAAGCCCCATCTCCA	140
GRINA	NM_153308	CATGGGAACTACCAGGAGGA	GTCAGCACCAGGAACACCTT	119
KLF9	NM_057211	AAGCTCCGCAGCCACCCTCA	CGGCGCGGTTGGAGATGGAA	125
MRAS	NM_012981	AGCCCAGTTCCCAGTCTCTT	TTGTCTGGTGCGTTGAATGT	101
NF1	NM_012609	GGCACTTCAAACCAAGCCGCA	GGAAGACCAGCGAGGAGCGAG	101
NINJ2	NM_021595	ATGCCCCTGCCTCTCACCCA	TGCAGTGCAACCAGAGTGCC	150
PPP1CA	NM_031527	AACGAGATCCGTGGTCTTTG	CTCGAACAGCCGTAGAAGGT	135
PPP1R14A	NM_130403	GGCAGACATGCCAGATGAG	CAGGCAAGCCTCCAAGAGT	134
PTPRF	NM_019249	CAGCTCCTCTGAGCCATACC	GGTGTTCTGGGCTCTCTGAC	103
REST	NM_031788	ATGGCGAGGCAGGAGCAGGT	TTGGCAGTGGTGACGGTGGG	118
VIP	NM_053991	AGACCCAAGGAGGCACCGAGA	TAGAGAGGCCAGGCCAGCGA	106
GLI1	XM_345832	TTCCATATCAGAGCCCCAAG	ATGCAAAGCCAGATCCAAAC	105
RPL36AL	NM_031105	GCCTCACAAAGTGACCCAGT	TGTGGTTTTGGCCTTCTTTC	133

Results

1. Blood and brain nicotine and cotinine concentrations in adolescent and adult animals

The concentrations of nicotine and cotinine achieved in plasma and brain are shown in Fig 2. Based on the weight changes that occurred during the 14 day dosing period (+10% in adults, +92% in adolescents), the nominal dose of free base nicotine changed from 6.0 to 3.1 mg/kg/day in adolescents, and to 5.5 mg/kg/day in adults. Accordingly, by the end of the 14 day dosing period, the plasma levels of both nicotine and its primary metabolite cotinine in adult rats were higher than those found in periadolescent rats (1.8-fold for nicotine, 2.2-fold for cotinine). A previous study using this same dosing protocol found similar weight changes, but reported lower absolute plasma levels of both compounds than found in this study, and found a greater discrepancy between levels in adults and adolescents (roughly 3–4 fold greater in adults) (Trauth et al., 2000b). The levels of nicotine were higher in brain than in blood, and were 2.2 times higher in adult brains compared to those of periadolescents. Our results are consistent with a previous study demonstrating that nicotine (but not cotinine) preferentially accumulates in brain versus blood over continual dosing regimens (Ghosheh et al., 2001).

Because this treatment protocol relies upon initial body weight for dose calculations, and because adolescents grow at a faster rate during the two-week dosing period, it was not surprising to find that by the end of the period, the actual per-weight dose in adolescents, as well as the corresponding blood levels of both nicotine and

cotinine, were approximately half that in adults; similar results were reported previously by Slotkin and colleagues (Trauth et al., 2000b) finding that brain nicotine levels in adults were also roughly twice those in adolescents is novel. While this difference is unrelated to differences in receptor expression between age groups, we cannot rule out that it contributes to differences in responsiveness to chronic nicotine exposure. Note, however, that the brain concentrations were quite high; even the lower concentration in adolescent brain would yield >99% occupancy of both α4β2* and α6* nAChRs, which suggests that the differences in concentration achieved may not be relevant to differential responsiveness of at least these two high-affinity receptor subtypes.

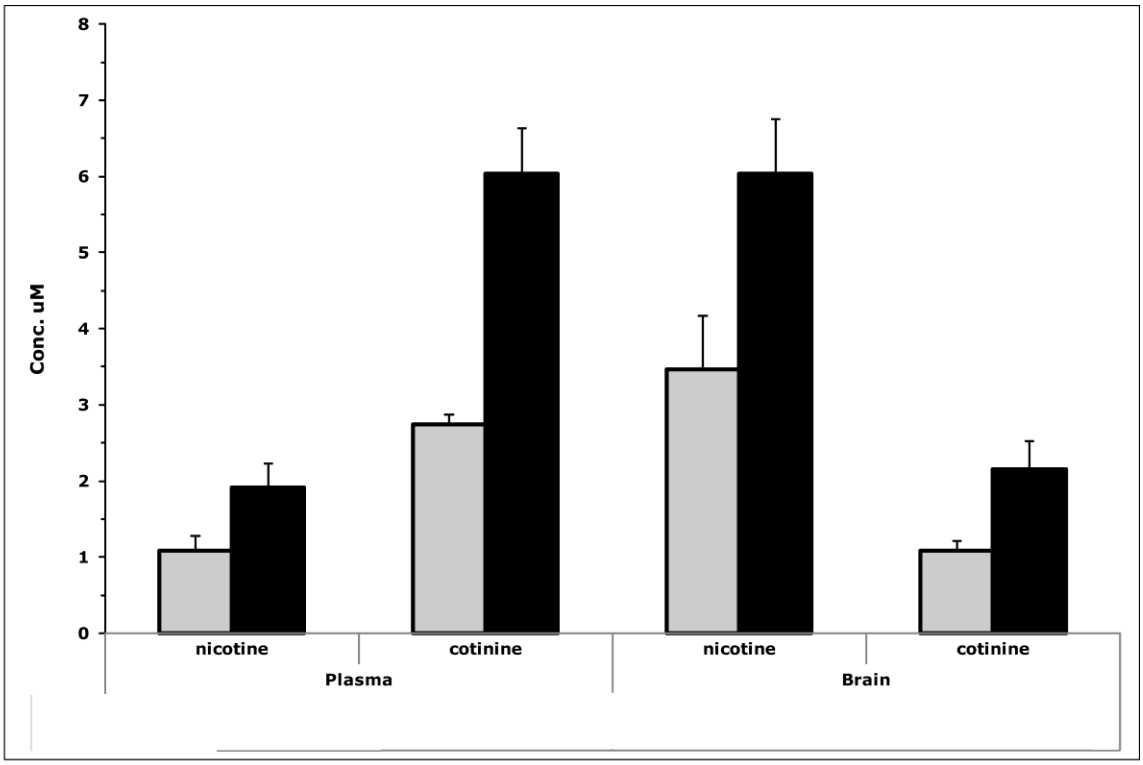

Figure 2. Plasma and brain nicotine and cotinine concentrations of adolescent and adult rats after 2 week nicotine treatment. Plasma nicotine P=0.0206; plasma cotinine P=0.003; brain nicotine P=0.0806; brain cotinine P=0.0209 (Student's t-test). Gray bars = adolescent, black bars = adult. Mean ± S.E.M. shown.

2. Radioligand binding to three major nAChR subtypes

Representative autoradiographic images are shown for [125I]A-85380 binding in Figure 3, for [125I]αBtx in Figure 4, and for [125I]α-CtxMII binding in Figure 5. Each figure shows total binding in equivalent regions from each of the four treatment groups: A, adult saline; B, adult nicotine; C, periadolescent saline; D, periadolescent nicotine. Non-specific binding in a section adjacent to one of the four total binding sections is shown in E. Adjacent sections from the same animals are shown across the three figures (e.g. 3A, 4A and 5A are all from the same adult saline-treated rat).

A-85380 has been shown to bind selectively to β2-containing nAChRs (Sullivan et al., 1996). The concentration of α-CtxMII included in the incubation (100 nM) should effectively block binding to α6β2* (Whiteaker et al., 2000; Champtiaux et al., 2002). The affinity of αCtxMII at α3β2* sites may be somewhat lower (Gotti et al., 2006) and the affinity at α2β2* sites is presently unknown. We will refer to the binding of [125I]A-85380 under these conditions as α4β2*, with the understanding that there may also be small contributions in some regions from these other two relatively minor subtypes. Visual evidence of up-regulated α4β2* binding is apparent in adults by comparing Figures 3A and 3B; in contrast, up-regulation of α4β2* binding in periadolescents is not as readily apparent by visual inspection of Figures 3C and 3D. [125I] αBtx labeling is widespread in mammalian brain, and is thought to be limited largely or entirely to α7 homomers. Similar distributions in adult and periadolescent brains are seen in Figure 4. Although some regions express high levels of both α4β2* and α7 nAChRs (e.g. superior colliculus, cerebral cortex), overall the pattern of α7 binding is very different from that

of α4β2*, with relatively high densities in hippocampus, amygdala and hypothalamus, and lower levels in striatum and thalamus compared to α4β2*.

At the concentration employed, [125I]α-CtxMII binding is selective for α6β2* nAChRs (Whiteaker et al., 2000; Champtiaux et al., 2002). Although αCtxMII was originally described in functional studies as selective for α3β2* nAChRs (Cartier et al., 1996), recent evidence suggests that its binding affinity to α3β2*is in the order of 50 nM (Gotti et al., 2006), which means that there would be very little contribution from α3β2* nAChRs in these studies, given the concentration of radioligand employed. Therefore, we will refer to the sites labeled by [125I]αCtxMII under the conditions employed here as α6* nAChRs. α6* nAChRs are expressed in visual systems from the retina to the superior colliculus, and in dopamine systems in rat brain (Whiteaker et al., 2000; Le Novere et al., 1996; Champtiaux et al., 2002). This distribution is reflected in the images shown in Figure 5, for both adult and periadolescent brains. No differences with nicotine treatment are readily apparent from comparing images.

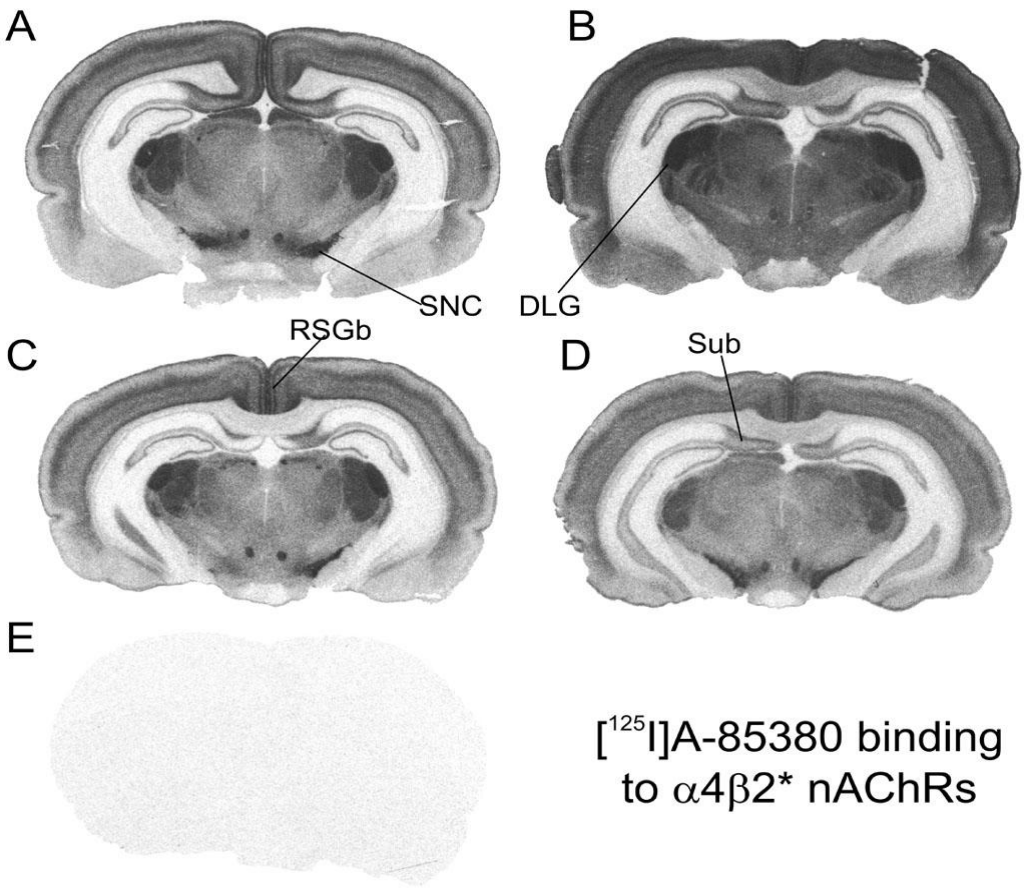

Figure 3. Autoradiographic images of [^{125}I]A-85380 binding to α4β2* nAChRs in rat brain sections from animals representing the four experimental groups

Binding of [^{125}I]A-85380 (0.6 nM) was done in the presence of 100 nM αCtxMII to block binding to α6/α3* subtypes, and is shown in representative sections from four experimental groups: **A**, adult saline; **B**, adult nicotine; **C**; periadolescent saline; **D**, periadolescent nicotine; **E**, non-specific binding in the presence of 100 µM nicotine in an adjacent section. DLG, dorsal lateral geniculate nucleus; RSGb, retrosplenial granular cortex; SNC, substantia nigra, pars compacta; Sub, subiculum. Sections cut approximately −5.0 mm from bregma.

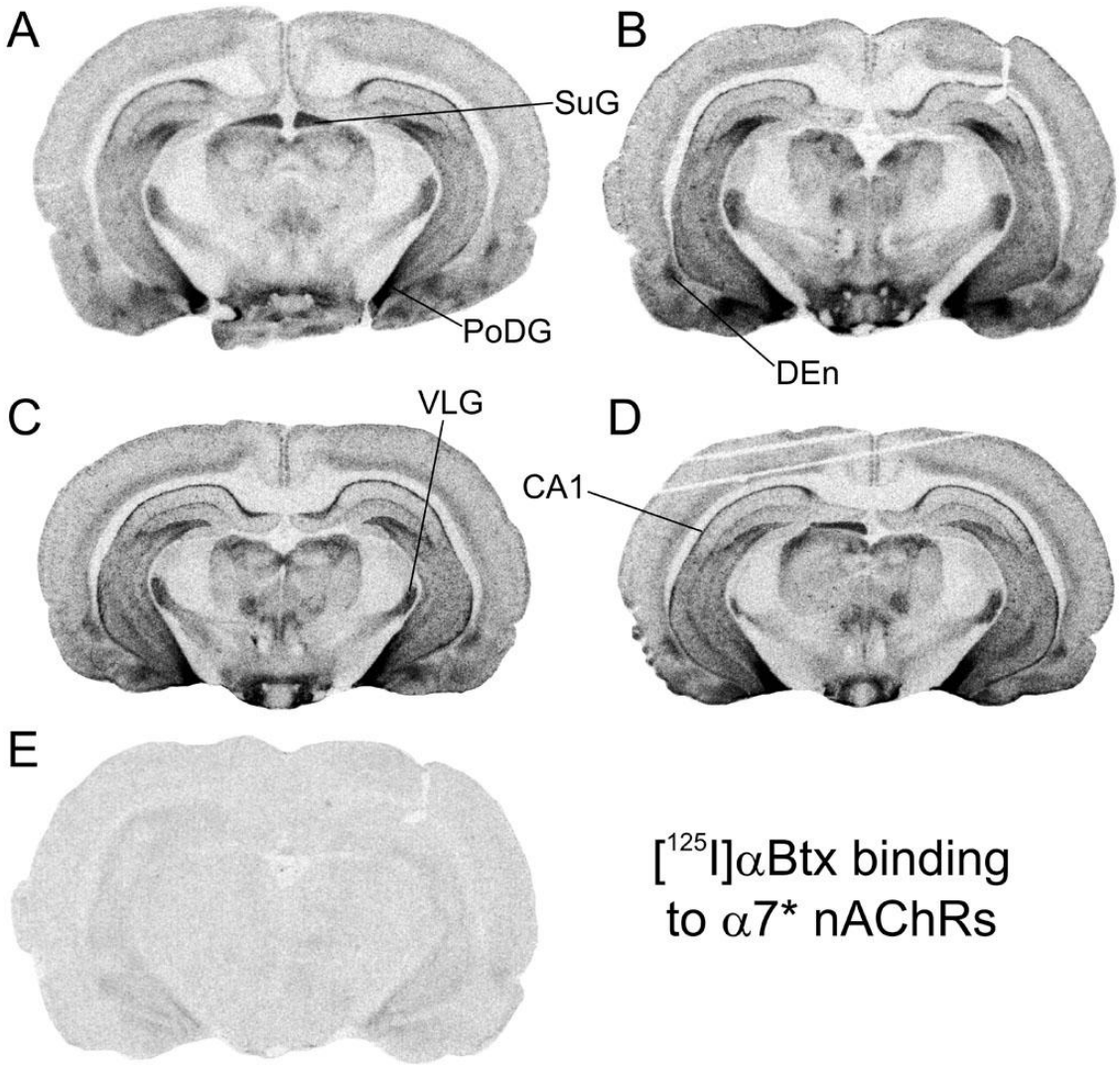

Figure 4. Autoradiographic images of [125I]αBtx binding to α7 nAChRs in rat brain sections from animals representing the four experimental groups

Binding of 0.72 nM [125I]αBtx is shown in representative sections from four experimental groups: **A**, adult saline; **B**, adult nicotine; **C**; periadolescent saline; **D**, periadolescent nicotine; **E**, nonspecific binding in the presence of 100 μM nicotine in an adjacent section. CA1, stratum oriens, hippocampus; DEn, endopiriform nucleus; PoDG, posterior dentate gyrus; SuG, superior colliculus, superficial grey layer; VLG, ventral lateral geniculate nucleus. Sections cut approximately −5.0 mm from bregma.

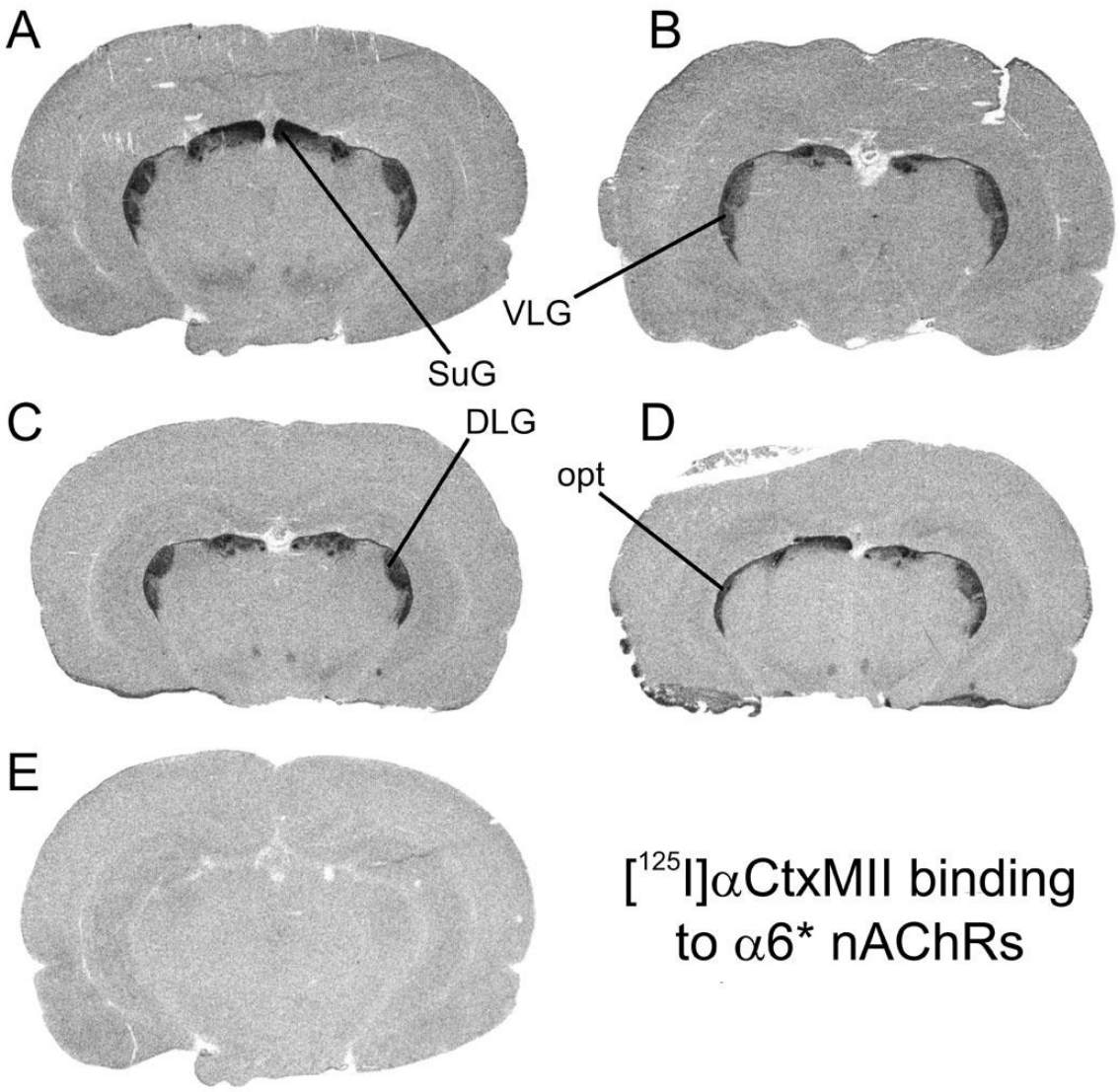

Figure 5. Autoradiographic images of [125I]αCtxMII binding to α6* nAChRs in rat brain sections from animals representing the four experimental groups

Binding of 0.8 nM [^{125}I]αCtxMII is shown in representative sections from four experimental groups: **A**, adult saline; **B**, adult nicotine; **C**; periadolescent saline; **D**, periadolescent nicotine; **E**, non-specific binding in the presence of 100 μM nicotine in an adjacent section. DLG, dorsal lateral geniculate nucleus; opt, optic tract; SuG, superior colliculus, superficial grey layer; VLG, ventral lateral geniculate nucleus. Sections cut approximately −5.0 mm from bregma.

3. α4β2* receptor numbers in saline treated animals

Binding was quantified by digital densitometry. Means of [125I]A-85380 binding to α4β2* receptors in 38 brain regions for the four treatment groups are shown in Table 2. (The individual images shown may not always reflect the quantitative results obtained by densitometry across multiple images.) Comparison of binding in adult versus periadolescent saline-treated controls reveals a distinct age effect: binding was uniformly higher in periadolescents. The mean binding across all 38 regions was 50% higher in periadolescents, with 33 of 38 regions demonstrating significantly higher binding in the younger rats. The comparison of binding in saline controls for adults and adolescents is presented graphically in Figure 6, demonstrating the age effects: adolescent binding as a percent of that in the adult.

Table 2. Binding (fmoles) of [^{125}I]A-85380 in the presence of 100 nM αCtxMII (to α4β2* nAChRs)

	Brain Region	Adults		Periadolescents		Treatment effects		Age effects	
		Saline	Nicotine	Saline	Nicotine	% chg from saline		% chg from adult	
		Mean +/-sem				Adult	Periadol.	Saline	Nicotine
1	Nu. accumbens, core	3.10±0.35	5.16±0.43	5.48±0.62	7.05±0.51	66.4**	28.5*	76.8**	36.5*
2	Nu. accumbens, shell	2.72±0.33	5.08±0.46	5.22±0.64	7.16±0.49	86.7**	37.2**	91.8**	40.9*
3	Endopiriform nu.	3.91±0.33	7.48±0.30	5.92±0.68	8.32±0.22	91.1***	40.4***	51.4***	11.3
4	Striatum, rostral	3.94±0.39	5.97±0.41	6.09±0.59	7.87±0.30	51.4**	29.2**	54.8**	31.8*
5	Striatum, caudal	3.39±0.35	5.36±0.50	5.40±0.66	7.13±0.48	58.3*	32.1	59.2*	32.9
6	Lateral septum	3.65±0.37	5.20±0.56	6.00±0.63	7.25±0.47	42.6*	20.8	64.6**	39.5*
7	Medial habenula	8.14±0.26	8.49±0.15	8.70±0.04	8.57±0.13	4.3	- 1.5	6.9	0.9
8	Fasiculus retroflexus	6.02±0.36	6.96±0.32	7.66±0.35	8.19±0.25	15.6	6.9	27.2**	17.7*
9	Posterior hypothalamus	1.29±0.18	4.38±0.65	2.06±0.32	5.41±0.71	239**	162***	59.4	23.3
10	Ventral posterior thal. nu.	6.12±0.44	8.21±0.32	7.54±0.41	8.50±0.21	34.2**	12.8	23.2*	3.5
11	Posterior thal. nu. group	6.91±0.41	8.46±0.24	8.08±0.27	8.63±0.13	22.4**	6.8	16.9**	2.0

12	Lateral post. thal. nu.	7.14±0.37	8.32±0.27	8.14±0.26	8.59±0.14	16.6*	5.6	14.1*	3.3
13	Internal capsule	1.52±0.17	2.32±0.33	4.12±0.71	4.82±0.66	52.6	16.8	171*	107*
14	Optic tract	1.92±0.20	2.00±0.32	4.58±0.87	5.28±0.63	4.6	15.2	139*	163***
15	Optic chiasm	5.88±0.45	6.98±0.39	7.43±0.44	8.16±0.28	18.7	9.9	26.4*	16.9
16	Superior collic., super. grey	6.11±0.54	7.29±0.30	7.56±0.42	8.09±0.32	19.5	7.0	23.7	10.8
17	Dorsal lateral geniculate	7.91±0.27	8.78±0.04	8.47±0.15	8.65±0.12	11.0	2.2	7.1	- 1.4
18	Ventral lateral geniculate	5.67±0.60	6.32±0.30	6.70±0.59	7.71±0.46	11.5	15.1	18.2	22.2
19	Medial geniculate	6.24±0.45	8.07±0.22	7.71±0.39	8.39±0.23	29.3**	8.9	23.4**	4.0
20	Zona incerta	2.44±0.29	5.84±0.67	4.63±0.66	6.96±0.55	140**	50.2*	90.0*	19.1
21	Tectal nuclei	5.44±0.56	6.02±0.30	6.72±0.52	7.46±0.47	10.7	11.1	23.6*	24.1
22	Substantia nigra, P.C.	6.07±0.75	7.44±0.55	8.06±0.26	8.31±0.28	22.6	3.0	32.8*	11.6
23	Ventral tegmental area	4.88±0.60	6.05±0.56	6.89±0.52	7.99±0.32	24.1	16.1	41.3*	32.2*
24	Dentate gyrus, hipp.	2.35±0.31	5.00±0.61	4.27±0.62	7.09±0.44	112*	66.0	81.5*	41.8*
25	CA1, hippocampus	1.21±0.13	3.29±0.48	2.29±0.37	5.06±0.68	171*	120***	89.1	53.7*
26	Subiculum	3.61±0.42	4.93±0.29	5.79±0.62	6.52±0.56	36.5	12.7	60.2*	32.3
27	Postsubiculum	7.23±0.37	8.25±0.16	8.37±0.18	8.60±0.14	14.1**	2.8	15.7**	4.3

28	Pontine nu.	2.40±0.47	3.98±0.44	4.11±0.77	5.99±0.67	66.1	45.6	71.7	50.5
29	Entorhinal cortex	3.01±0.37	6.50±0.24	5.15±0.70	7.33±0.50	116***	42.3*	71.1**	12.8
30	Frontal cortex, L4	4.08±0.40	6.59±0.50	6.04±0.59	7.83±0.38	61.6**	29.7*	48.1**	18.9
31	Frontal cortex, L5	3.10±0.32	6.70±0.38	5.20±0.66	7.58±0.44	116***	45.6**	67.9**	13.0
32	Cingulate cortex, outer	5.48±0.40	7.09±0.36	7.35±0.46	8.19±0.28	29.3**	11.4	34.3**	15.6
33	Cingulate cortex, inner	4.89±0.43	7.13±0.38	6.91±0.54	8.45±0.16	45.9**	22.2*	41.4**	18.5*
34	Retrosplenial cortex, L1-2	6.79±0.41	8.04±0.18	8.07±0.33	8.57±0.14	18.4**	6.2	18.8**	6.6
35	Retrosplenial cortex, L3-4	6.36±0.44	7.93±0.18	7.93±0.37	8.44±0.19	24.7**	6.5	24.6**	6.5
36	Visual cortex, L4	4.95±0.50	7.54±0.31	6.70±0.61	8.11±0.32	52.4**	21.0	35.5*	7.6
37	Visual cortex, L5	3.42±0.44	6.44±0.53	5.60±0.68	7.72±0.42	88.0**	37.9*	63.4**	19.8
38	Visual cortex, L6	4.47±0.44	6.73±0.32	6.53±0.63	7.87±0.37	50.7**	20.5	46.1**	16.9

Means determined from 5-8 replicates. Significance of comparisons: saline vs. nicotine treatment, or adult vs. periadolescent age;

*p<0.05; **p<0.01; ***p<0.001.

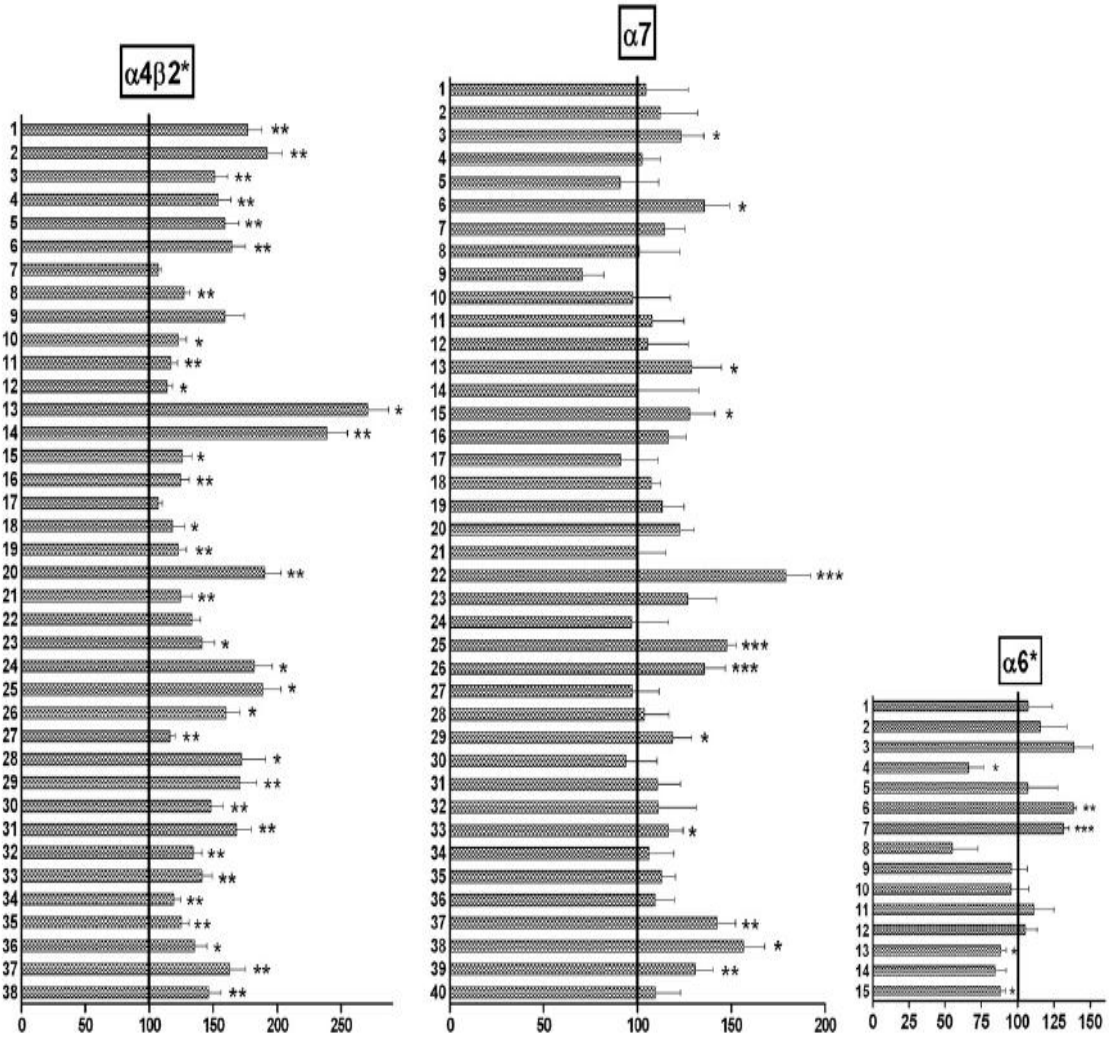
α4β2*
α7
α6*

Figure 6. Binding in control adolescent brains as a % of that in control adult brains

Age effects: Binding to three nAChR subtypes compared in saline controls from adults and periadolescent rats. Binding in periadolescent brains is shown as a percent of that in the equivalent brain region in the adult animal; the 100% line indicates where binding would be if it was equal in adults and periadolescents. Numbers refer to specific brain regions identified in Table 2 (for α4β2* nAChRs), Table 3 (for α7 nAChRs) or Table 4 (for α6* nAChRs). Different from binding in the equivalent brain region in adults: *p<0.05; **p<0.01; ***p<0.001; 2-way ANOVA with Holm–Sidak post-test.

4**. α4β2* receptor numbers in nicotine treated animals**

A similar but less pervasive pattern was seen when comparing nicotine-treated adults and periadolescents: higher binding in younger rats. As reported in previous studies, there was widespread up-regulation of α4β2* binding in adult rats: the average binding across 38 regions was 55% higher after nicotine treatment, and 28 of 38 regions showed a statistically significant increase (none were lower). The increases in α4β2* binding with chronic nicotine were not uniform across regions: large and consistent increases were detected across cerebral cortex, and in many forebrain regions and hippocampus; effects were much more varied in diencephalon and midbrain structures. The effect of nicotine exposure on α4β2* receptors in periadolescents was noticeably smaller. The average binding across 38 regions was 27% higher after nicotine treatment, and only 13 of 38 regions showed a statistically significant increase (none were significantly decreased). The increase was larger in adults than in periadolescents for 35 of the 38 regions (for the remaining three, the larger increase in periadolescents was non-significant). The treatment effects of nicotine on binding in adults and adolescents are presented graphically in Figures 7 and 8, showing binding in nicotine-treated animals as a percent of that in saline-treated animals. To obtain a more global sense of the effects of age and treatment on α4β2* binding, we performed GLM analysis on the means collapsed across all regions measured. There was no interaction between age and treatment ($F=0.17$; $p=0.6861$). The overall effects of age ($F=11.45$; $p=0.0021$) and nicotine treatment ($F=11.37$; $p=0.0022$) were both highly significant for binding to the α4β2* nAChR.

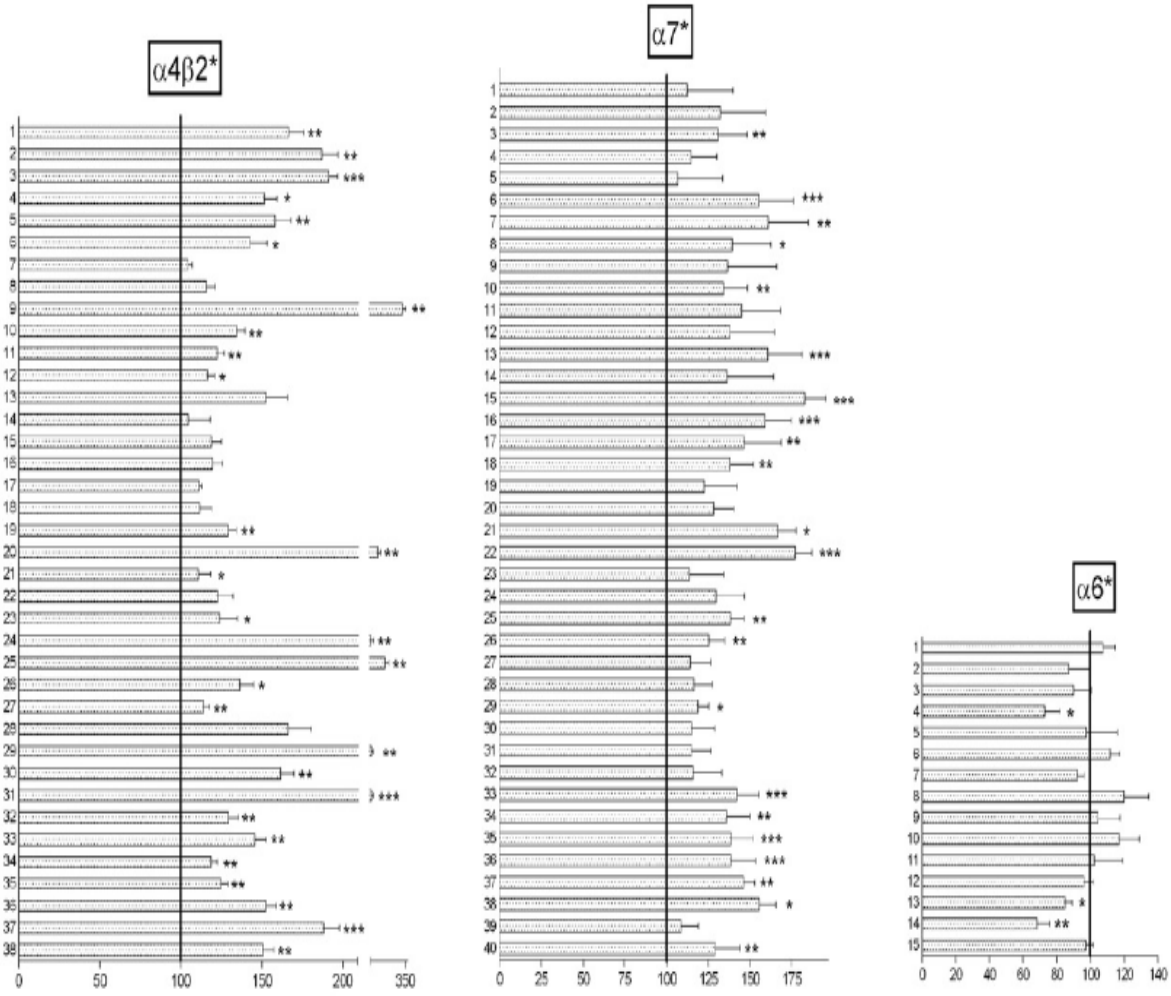
α4β2*
α7*
α6*

Figure 7. Binding in nicotine treated adult animals as a % of saline controls

Treatment effects: binding to three nAChR subtypes in adult rat brains. Binding in nicotine-treated animals is shown as a percent of that in the equivalent brain region in the saline-treated adult animals. The 100% line indicates where the binding would be if it was unaffected by nicotine treatment. Left graph shows binding to α4β2* nAChRs; middle graph shows binding to α7 nAChRs; right graph shows binding to α6* nAChRs. Numbers on Y-axis refer to specific brain regions identified in Table 2 (for α4β2*), Table 3 (for α7) and Table 4 (for α6*). Different from binding in the equivalent brain region in saline controls: *$p<0.05$; **$p<0.01$; ***$p<0.001$; 2-way ANOVA with Holm–Sidak post-test.

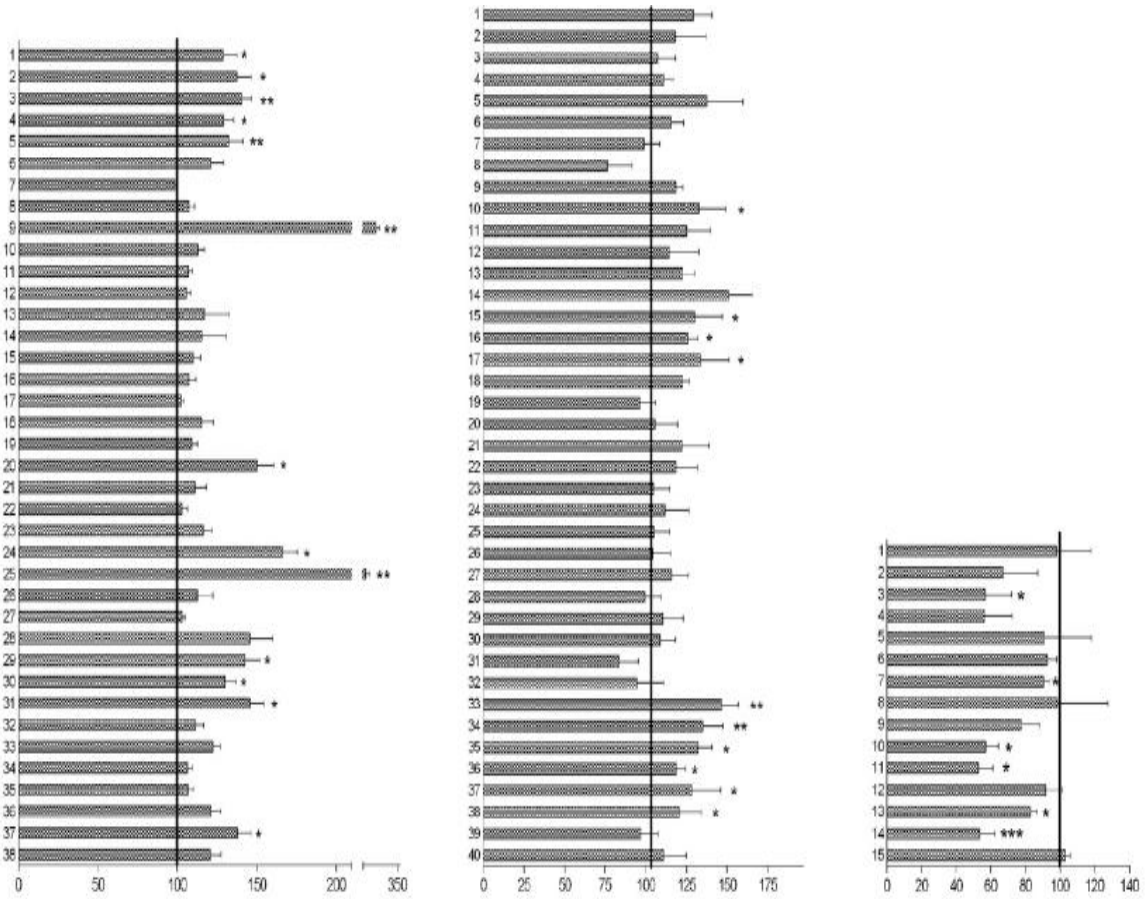

Figure 8. Binding in nicotine treated adolescent animals as a % of saline controls

Treatment effects: binding to three nAChR subtypes in adult and periadolescent rat brains. Binding in nicotine-treated animals is shown as a percent of that in the equivalent brain region in the saline-treated animals. The 100% line indicates where the binding would be if it was unaffected by nicotine treatment. The left graph shows binding to α4β2* nAChRs; the middle graph shows binding to α7 nAChRs; the right graph shows binding to α6* nAChRs. Numbers on Y-axis refer to specific brain regions identified in Table 2 (for α4β2*), Table 3 (for α7) or Table 4 (for α6*). Different from binding in the equivalent brain region in saline controls: *$p<0.05$; **$p<0.01$; ***$p<0.001$; 2-way ANOVA with Holm–Sidak post-test.

5. α7 binding in saline treated animals

Comparison of α7 binding in adult versus periadolescent saline-treated controls reveals similar but less dramatic age-related differences (Table 3; Figure 6). The mean binding across all 40 regions was 15% higher in saline-treated periadolescents compared to saline-treated adult rats; with 12 of 40 regions measured demonstrating significantly higher binding in the younger rats (none were significantly lower in periadolescents).

6. α7 binding in nicotine treated animals

As previously reported, chronic nicotine also causes up-regulation of α7 binding in adult rats (Rasmussen & Perry, 2006) and mice (Pauly et al., 1991) although to a lesser degree and in fewer regions than occurs with α4β2* nAChRs. Across all 40 regions, there was a mean binding increase of 36% in adult rats, with 20 of 40 regions demonstrating significant increases; no region showed significantly lower binding after nicotine (Table 3; Figures 7 & 8). Up-regulation was most prominent in cerebral cortex, with several regions also up-regulated in hippocampus, hypothalamus and amygdala. As with α4β2*, the nicotine effect was much smaller in periadolescents. The mean increase was 16%, and only 10 of 40 regions showed significant increases (none showed significant decreases). To obtain a more global sense of the effects of age and treatment on α7* binding, we performed GLM analysis on the means collapsed across all regions measured. There was no interaction between age and treatment ($F=2.32$; $p=0.1402$). The overall effect of age was not significant ($F=2.41$; $p=0.1334$), but the overall effect of nicotine treatment was significant ($F=9.62$; $p=0.0047$).

Table 3. Binding (fmoles) of [^{125}I]α-bungarotoxin (to α7 nAChRs).

	Brain Region	Adults		Periadolescents		Treatment effects		Age effects	
		Saline	Nicotine	Saline	Nicotine	% chg from saline		% chg from adult	
		Mean +/-sem				Adult	Periadol.	Saline	Nicotine
1	Striatum	1.51±0.46	1.70±0.43	1.58±0.24	2.04±0.17	12.6	29.4	4.5	20.0
2	Nucleus accumbens	1.78±0.48	2.35±0.64	1.99±0.27	2.36±0.55	32.5	18.3	12.1	0.1
3	Septum	6.03±1.04	7.90±1.34	7.43±0.63	7.97±1.07	31.0	7.2	23.2	0.8
4	Nu. vertical limb diag. band	5.65±0.93	6.47±0.99	5.79±0.19	6.43±0.52	14.6	11.2	2.5	-0.6
5	Ventral pallidum	3.79±1.01	4.06±1.07	3.45±0.47	4.74±1.37	7.0	37.6	- 9.1	16.9
6	Endopiriform nu.	10.7±1.99	16.7±3.59	14.5±1.39	16.9±0.92	55.6***	15.9	35.7*	1.1
7	Bed nu., stria terminalis	5.77±0.86	9.29±2.75	6.58±0.50	6.50±0.77	61.1**	-1.1	14.1	-30.0*
8	Anterior hypothalamus	9.67±2.52	13.5±2.86	9.79±1.61	7.47±1.01	39.5*	-23.7	1.3	-45**
9	Supraoptic nu. hypothalamus	26.1±3.67	35.6±14.5	18.5±1.43	21.9±0.36	36.7	18.4	-29.2	-38.6
10	Posterior hypothalamus	12.3±2.87	16.4±1.24	12.0±1.99	15.9±2.68	34.0**	32.8*	-2.4	-3.2
11	Lateral hypothalamus	4.85±1.35	7.03±1.44	5.23±0.33	6.54±1.43	44.9*	24.9	7.8	-7.1
12	Ventromedial hypothalamus	13.0±3.40	17.9±4.94	13.7±2.42	15.7±2.92	37.9	14.4	5.4	-12.6

13	Zona incerta	3.84±1.16	6.18±0.87	4.95±0.23	6.08±0.58	60.9***	22.7*	29.0*	-1.6
14	Subthalamic nu.	20.4±10.39	27.8±3.05	20.5±2.70	30.9±4.75	36.5	50.9*	0.5	11.0
15	Lateral amygdaloid nuclei	5.23±0.58	9.56±1.33	6.68±1.01	8.70±1.59	82.9***	30.2**	27.8	-9.0
16	Basal amygdaloid nuclei	17.7±2.74	28.2±4.61	20.7±0.90	26.1±1.88	59.1***	26.0**	16.6	-7.6
17	Posteromed. cort. amygdala	19.3±4.70	28.2±5.93	17.5±2.65	23.4±4.65	46.4*	33.6	-9.0	-17.0
18	Amygdalohippocampal area	18.1±1.11	24.9±5.08	19.4±0.88	23.8±0.92	38.0*	22.6*	7.2	-4.7
19	Stratum oriens, hippocampus	7.58±1.23	9.28±2.11	8.57±0.64	8.26±0.98	22.5	-3.5	13.0	-11.0
20	S. lac.-molec., hippocampus	5.55±0.44	7.12±1.11	6.79±0.51	7.20±1.40	28.4*	6.0	22.5	1.1
21	Dentage gyrus, hippocampus	11.3±1.46	18.9±1.77	11.4±1.82	13.9±2.47	66.8***	22.1	0.6	-26***
22	Ventral CA1, hippocampus	6.18±0.80	10.9±0.93	11.1±1.43	13.1±1.80	77.2***	18.4*	79.1**	19.6*
23	Posterior dentate gyrus	58.8±12.38	66.9±13.6	74.5±8.03	77.8±7.45	13.8	4.5	26.7	16.4
24	Parasubiculum	15.1±2.89	19.6±2.96	14.6±2.96	16.3±1.65	29.8*	11.8	-3.1	-16.6
25	Tectal nuclei	8.41±0.22	11.7±1.40	12.4±0.82	13.0±1.69	38.5***	4.9	47.6***	11.8
26	Ventral lateral geniculate	11.1±1.57	13.9±0.89	15.1±1.34	15.7±2.13	25.2**	4.0	35.7**	12.7
27	Superior collic.,super. grey	19.7±2.83	22.4±2.34	19.1±2.70	22.2±1.46	14.2	16.1	-2.7	-1.1
28	Superior collic., optic L	11.4±1.81	13.4±0.90	11.9±1.19	11.8±1.19	16.8	-0.7	3.8	-11.7

29	Mammillary nu.	19.5±1.23	23.2±1.56	23.1±3.15	25.5±3.05	18.9*	10.3	18.7*	10.1
30	Dorsal raphe nu.	11.7±2.78	13.5±0.73	11.0±1.08	12.0±1.06	15.0	8.9	-6.3	-11.3
31	Parabigeminal nu.	15.4±2.46	17.7±1.49	17.0±1.50	14.3±2.16	14.8	-16.1	10.7	-19.1*
32	Microcellular tegmental nu.	12.3±2.43	14.2±2.10	13.6±2.73	12.9±1.68	16.1	-5.3	11.2	-9.3
33	Frontal cortex, inner laminae	5.76±0.49	8.20±1.31	6.72±0.46	9.84±1.24	42.4***	46.4**	16.6	19.9*
34	Frontal cortex outer laminae	3.73±0.59	5.08±0.66	3.96±0.41	5.35±0.72	36.0**	35.1**	6.2	5.4
35	Cingulate cortex, lateral	6.02±0.75	8.34±1.16	6.79±0.20	8.99±1.11	38.7***	32.4*	12.9	7.8
36	Cingulate cortex, medial	4.79±0.82	6.64±0.87	5.24±0.20	6.23±0.43	38.7***	18.8	9.4	-6.2
37	Visual cortex, inner laminae	3.15±0.15	4.61±0.37	4.50±0.59	5.77±1.21	46.3*	28.5*	42.6*	25.2*
38	Visual cortex, outer laminae	3.92±0.42	6.11±0.58	6.14±0.70	7.41±1.13	55.7***	20.6*	56.5**	21.3*
39	Retrosplenial cortex	5.18±0.67	5.64±0.43	6.76±0.48	6.56±0.90	9.0	-3.0	30.6**	16.2*
40	Entorhinal cortex	10.4±1.41	13.5±2.14	11.4±1.52	12.7±1.83	29.3*	10.8	9.5	-6.1

Means determined from 5-8 replicates. Significance of comparisons: saline vs. nicotine treatment, or adult vs. periadolescent age;

*p<0.05; **p<0.01; ***p<0.001.

7. α6 binding in saline treated animals

Comparison of [125I]α-CtxMII in adult versus periadolescent saline-treated controls did not show system-wide age-related differences (Table 4; Figure 6): the mean binding across 15 regions in periadolescents was 2% higher than in adults. Two regions (optic chiasm, optic tract) were significantly greater in periadolescents, while three (caudal striatum, dorsal lateral geniculate, superior colliculus) were significantly lower.

8. α6 binding in nicotine treated animals

The comparison across age in nicotine treated animals showed that six regions were significantly lower in periadolescents, while one was higher (optic tract). In contrast to α4β2* and α7 nAChRs, autoradiographic studies have found that chronic nicotine causes either no change or a decrease in binding to α6* nAChRs (Perry et al., 2007; Mugnaini et al., 2006; Lai et al., 2005). In the current studies we also found a trend towards decreased binding with chronic nicotine exposure: a 5% average decrease across 15 regions; binding was significantly decreased in three regions, increased in none (Table 4; Figure 7 & 8). In contrast to the other two nAChR subtypes, the effect of chronic nicotine was more pronounced in periadolescents than adults: across all 15 regions, there was a mean decrease of 21%, and six of 15 regions in periadolescents showed a significant decrease with nicotine exposure. To obtain a more global sense of the effects of age and treatment on α6* binding, we performed GLM analysis on the means collapsed across all regions measured. There was no significant interaction between age and treatment ($F=3.68$; $p=0.0653$). The overall effect of age was not

significant (F=0.23; p=0.6352), but the overall effect of nicotine treatment was significant (F=3.68; p=0.0044).

Table 4. Binding (fmoles) of [^{125}I]α-conotoxin MII (to α6/α3* nAChRs).

	Brain region	Adults		Periadolescents		Treatment effects		Age effects	
		Saline	Nicotine	Saline	Nicotine	% chg from saline		% chg from adults	
		Mean +/-sem				Adult	Periadol.	Saline	Nicotine
1	Nu. accumbens, shell	0.11±0.01	0.11±0.01	0.12±0.03	0.11±0.02	+ 7.3	- 2.2	+ 7.3	+ 0.1
2	Nu. accumbens, core	0.19±0.03	0.16±0.02	0.21±0.05	0.14±0.02	- 13.0	- 33.0	+15.3	-11.1
3	Striatum, rostral	0.21±0.02	0.19±0.02	0.29±0.05	0.17±0.02	- 10.3	- 43.1*	+38.7	-11.9
4	Striatum, caudal	0.31±0.02	0.22±0.03	0.20±0.03	0.11±0.02	- 27.2*	- 43.8*	- 33.3**	-48.9*
5	Olfactory tubercle	0.14±0.03	0.14±0.02	0.15±0.03	0.14±0.05	- 2.7	- 8.9	+7.0	+ 0.2
6	Optic chiasm	1.64±0.04	1.84±0.15	2.27±0.06	2.11±0.17	+11.6	- 7.3	+38.2*	+14.7
7	Optic tract	1.84±0.07	1.69±0.08	2.42±0.09	2.19±0.06	- 8.1	- 9.5	+31.6**	+29.7
8	Medial habenula	0.26±0.03	0.31±0.05	0.14±0.04	0.14±0.04	+19.9	- 1.8	- 45.1	-55.1*
9	Fasiculus retroflexus	0.40±0.05	0.42±0.06	0.38±0.03	0.30±0.03	- 22.3	+ 4.4	- 4.1	-28.5
10	Ventral tegmental area	0.28±0.04	0.32±0.03	0.26±0.02	0.15±0.01	+16.9	- 43.0*	- 4.4	-53.4
11	Substantia nigra, PC	0.28±0.05	0.29±0.05	0.32±0.03	0.17±0.01	+ 2.1	- 47.4*	+11.4	-42.6

12	Tectal nuclei	1.82±0.13	1.75±0.07	1.92±0.17	1.76±0.17	- 3.9	- 8.3	+ 5.5	+ 0.7
13	Dorsal lateral geniculate	1.95±0.07	1.66±0.08	1.73±0.07	1.43±0.05	- 15.1*	- 17.1*	- 11.6*	-13.7
14	Ventral lateral geniculate	1.84±0.12	1.26±0.11	1.56±0.13	0.84±0.08	- 31.8*	- 46.4**	- 15.4	-33.5*
15	Superior collic. super. grey	3.27±0.14	3.18±0.14	2.88±0.10	2.97±0.06	- 2.9	+ 2.8	- 12.1	- 6.6

Means determined from 5-8 replicates. Significance of comparisons: saline vs. nicotine treatment, or adult vs. periadolescent age;

*p<0.05; **p<0.01; ***p<0.001.

9. Age specific gene regulation in the VTA by nicotine

Whole genome expression was measured in the VTA of adolescent and adult male SD rats exposed to saline, chronic nicotine for 14 days, or 30 days withdrawal following chronic nicotine. A two-way ANOVA with 10% FDR revealed 4878 genes with a significant age X treatment interaction. We argue 10% FDR is favorable over more strict corrections in this application because the lists of significant genes will be subjected to several subsequent tests for biological significance. These subsequent analyses will further parse the gene lists for biologically relevant genes. Next, we applied template matching (also known as feature selection) (Pavlidis & Noble, 2001) to search for patterns of expression across age and treatment, identifying three distinct temporal expression patterns referred to as "transient response", "persistent response" and "late response" genes. Transient response genes displayed an initial response (up- or down-regulation) to chronic nicotine compared to saline/baseline as measured at the end of the two week treatment period, but had returned to baseline expression levels at the end of the 30 day withdrawal period (Fig. 9A and 9B). There were 80 adolescent-specific genes (Fig. 9A) and 171 adult-specific genes (Fig. 9B) that exhibited a transient response to chronic nicotine. Persistent response genes displayed an initial response to chronic nicotine as measured at the end of the two week treatment period, and remained up- or down-regulated at the end of the 30 day withdrawal period (Fig. 9D and 9E). Sixty two adolescent-specific genes (Fig. 9D) and 33 adult-specific genes (Fig. 9E) exhibited a persistent pattern of response. Late response genes showed no initial response to chronic nicotine at the end of the two week treatment period, but showed significant

up- or down-regulation at the end of the 30 day withdrawal period (Fig. 9G and 9H). A total of 532 adolescent-specific genes (Fig. 9G) and 101 adult-specific genes (Fig. 9H) exhibited a late response to chronic nicotine.

10. Shared genes regulated by nicotine

A one-way ANOVA with 10% FDR revealed 973 adult genes and 3072 adolescent genes that were significant for chronic nicotine regulation. Of these, 425 genes were shared between the two age groups. Eleven shared genes exhibited a transient response (Fig. 9C), 9 shared genes exhibited a persistent response (Fig. 9F), and 352 shared genes exhibited a late response to chronic nicotine (Fig. 9I). It is noteworthy that the majority of shared gene regulation (≈83%) occurred after the end of chronic nicotine treatment (late response shared genes). By comparison, the number of transient and persistent response shared genes accounted for less than 6% of all shared genes (Fig. 10).

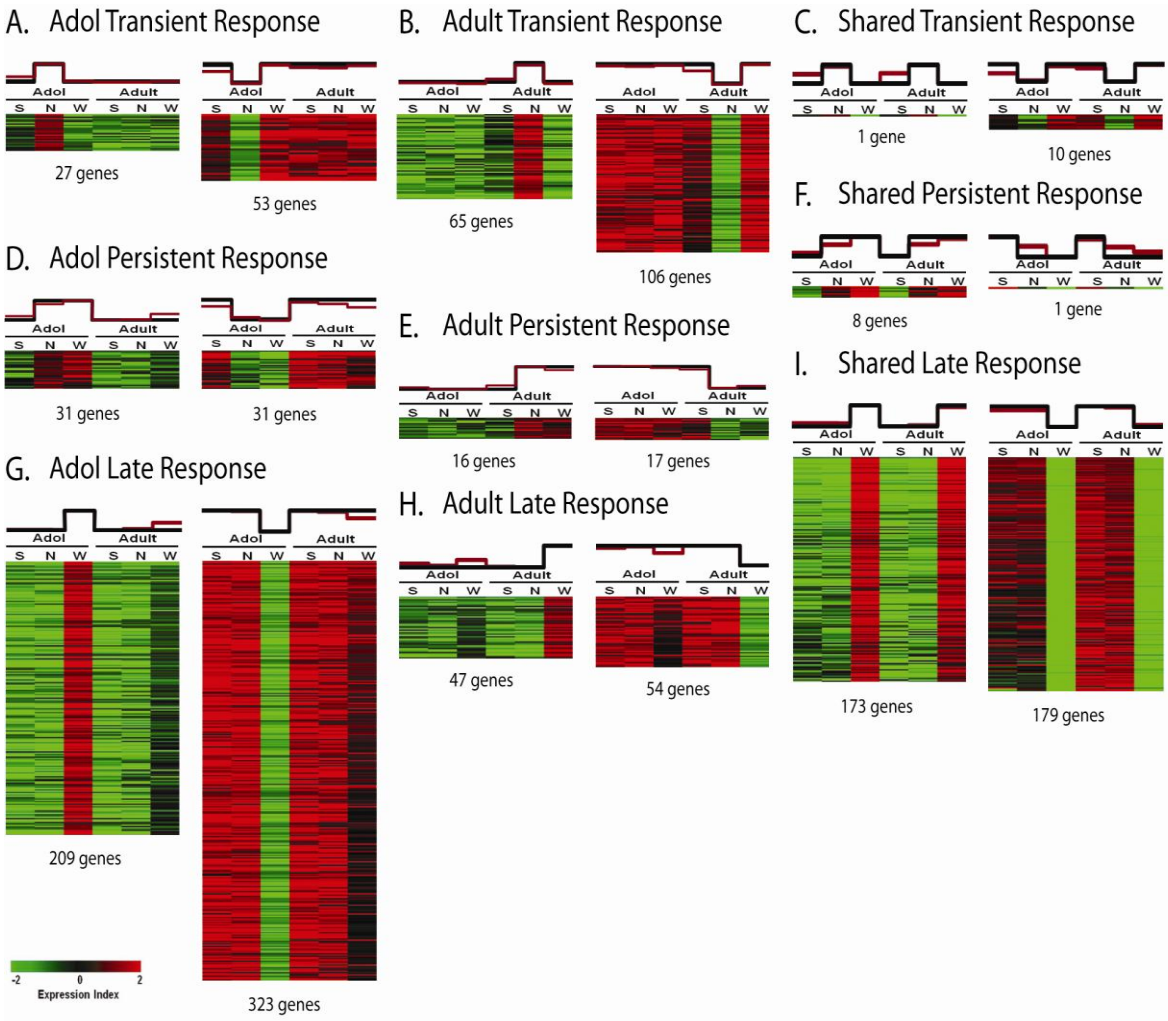
A. Adol Transient Response
Adol
Adult
S N W S N W
27 genes
53 genes
B. Adult Transient Response
65 genes
106 genes
C. Shared Transient Response
1 gene
10 genes
D. Adol Persistent Response
31 genes
31 genes
E. Adult Persistent Response
16 genes
17 genes
F. Shared Persistent Response
8 genes
1 gene
G. Adol Late Response
209 genes
323 genes
-2 0 2
Expression Index
H. Adult Late Response
47 genes
54 genes
I. Shared Late Response
173 genes
179 genes

Figure 9. Gene expression correlation with chronic nicotine exposure. Heat maps of template-matched genes show positive and negative correlations with chronic nicotine treatment. The black line above each heat map represents the specific template employed for matching, whereas the red line represents the mean expression values of all matched genes within each column. Each pattern includes both a positive template (corresponding to gene up-regulation, left) and a negative template (corresponding to gene down-regulation, right). **A**. Genes showing a transient response to chronic nicotine in adolescent rats. **B**. Genes showing a transient response to chronic nicotine in adult rats. **C**. Genes showing a transient response to chronic nicotine shared between adolescent and adult rats. **D**. Genes showing a persistent response to chronic nicotine in adolescent rats. **E**. Genes showing a persistent response to chronic nicotine in adult rats. **F**. Genes showing a persistent response to chronic nicotine shared between adolescent and adult rats. **G**. Genes showing a late response to chronic nicotine in adolescent rats. **H**. Genes showing a late response to chronic nicotine in adult rats. **I**. Genes showing a late response to chronic nicotine shared between adolescent and adult rats.

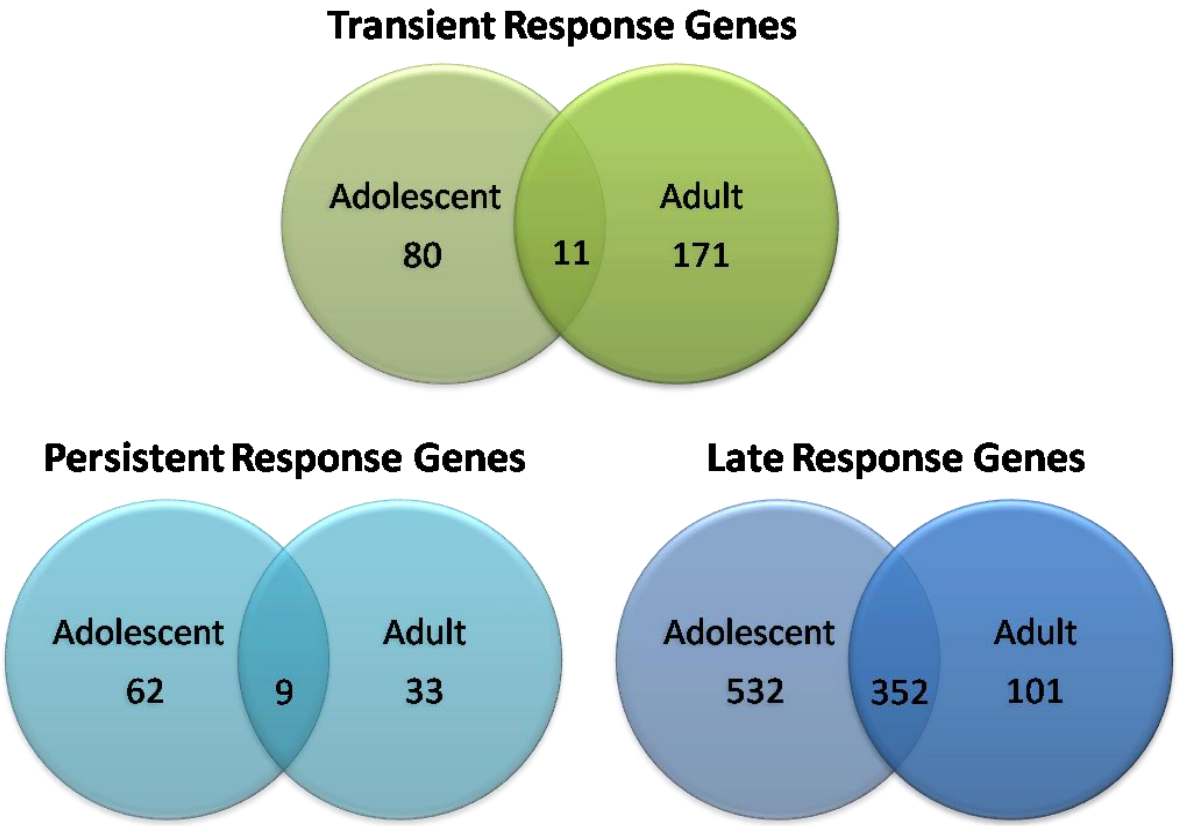

Figure 10. Venn diagram of template-matched genes significantly regulated by chronic nicotine treatment in adolescent and adult rats.

11. Age specific biological functions overrepresented in VTA of nicotine treated animals

Adolescent- and adult-specific genes (transient, persistent and late response) were further analyzed for biological significance. Ingenuity Pathway Analysis (IPA) was employed for the identification of top ranked biological functions, significant networks, and canonical pathways associated with these age-specific genes. Figure 11 shows high level biological functions identified by IPA that were significantly (Fisher's exact test) overrepresented in our adolescent-specific, adult-specific, and shared data sets, including genetic disorders, psychological disorders, neurological disease and behavior.

Further analysis focused on genes that contribute to nervous system development and function. This category consisted of 42 adolescent-specific, 14 adult-specific, and 23 shared genes involved in a number of biological processes (Table 5). Figure 12 depicts an interaction network of adolescent- (Fig. 12A) and adult-specific genes (Fig. 12B) involved in nervous system development and function. The adolescent-specific genes form an extensive interaction network in contrast to adult-specific genes.

Our study has identified a large number (121) of genes related to neurological disorders significantly regulated by adolescent nicotine exposure that are unchanged when nicotine is given during adulthood. More specifically, expression of 26 genes relating to schizophrenia was changed in adolescent nicotine-treated rats but not in their adult counterparts. No schizophrenia-related genes were identified specific to the adult nicotine treated population. We also identified a number of genes related to Huntington's disease that are significant for regulation by nicotine: 25 adolescent

specific genes and 15 adult specific genes were identified involved in Huntington's disease. Taken together, these data suggest a particular sensitivity of adolescent animals to nicotine exposure in regards to neurological and psychiatric disease. Exposure to chronic nicotine during adolescence activates pathways involved in the etiology of these disorders and may increase the risk of developing disease.

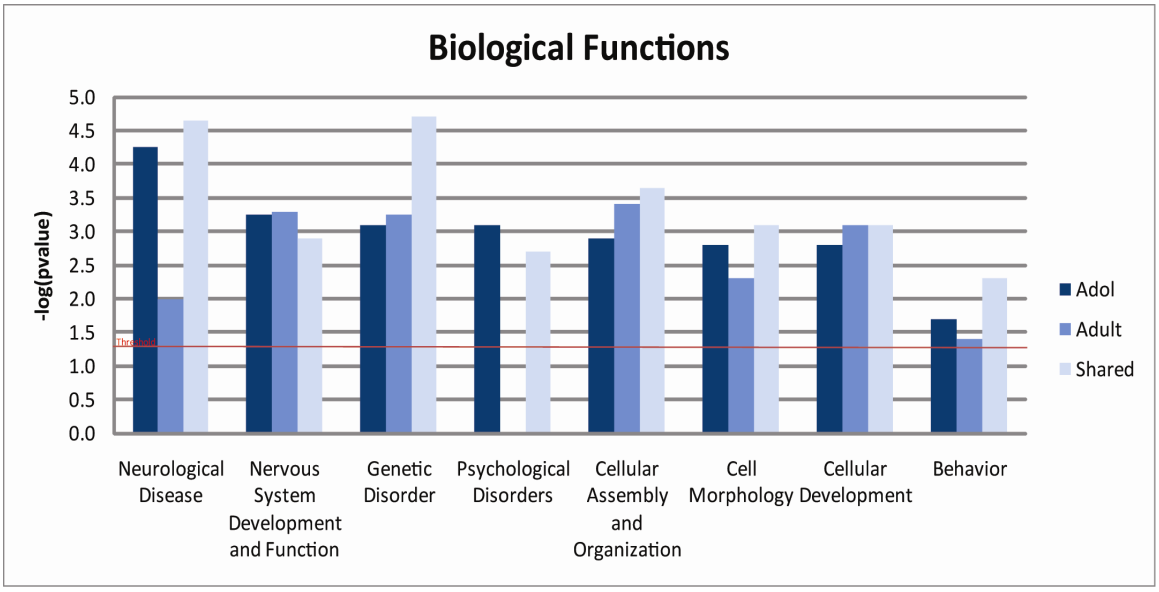

Figure 11. Over-represented biological functions with possible biological relevance to chronic nicotine treatment. These data were generated in IPA and significance is expressed as the inverse log of the p-value. The threshold line indicates $p < 0.05$ (Fisher's exact test).

Table 5. Numbers and functions of genes identified by Ingenuity Pathways Analysis involved in nervous system development and function in adolescent and adult animals including age-specific and shared genes.

	Adolescent		Adult	
Function	P-Value	# of Genes	P-Value	# of Genes
Development of brain	1.33E-02	16	—	—
Development of forebrain	1.95E-02	8	—	—
Development of neurites	2.65E-03	18	—	—
Growth of neurites	7.78E-03	24	—	—
Learning of mice	4.23E-02	5	1.10E-02	5
Length of dendrites	2.00E-02	3	—	—
Length of neurites	7.54E-03	6	—	—
Long term depression	9.52E-03	6	—	—
Morphogenesis of dendrites	2.72E-02	6	—	—
Morphogenesis of neurites	4.89E-03	11	—	—
Neurological process of axons	3.03E-02	11	—	—
Neurological process of cerebral cortex	4.23E-02	5	—	—
Neurological process of mice	2.59E-02	19	—	—
Outgrowth of neurites	1.38E-02	21	—	—
Quantity of presynaptic terminals	—	—	1.06E-04	3

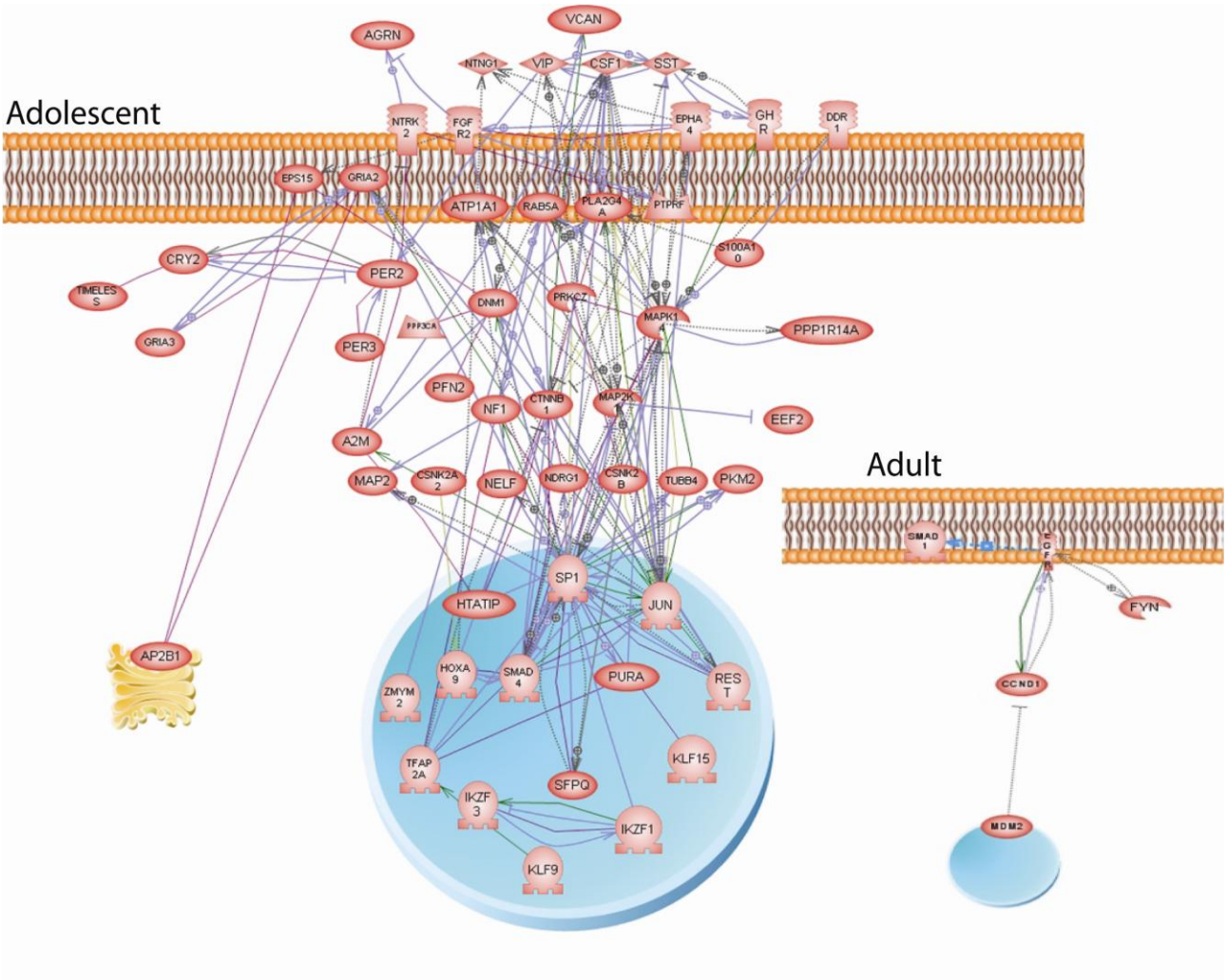

Figure 12. Interaction network of genes significant for regulation by chronic nicotine falling within Nervous System Development and Function. **A**. Adolescent nervous system and development network. **B**. Adult nervous system and development network.

12. Age specific canonical pathways in VTA of nicotine treated animals

Figure 13 depicts the canonical pathways identified by IPA as significantly over-represented (Fisher's exact test) in adolescent-specific, adult-specific or shared genes regulated by chronic nicotine treatment. Five of the seven canonical pathways shown are unique to either adolescents or adult animals. Of note is the synaptic long-term potentiation (LTP) canonical pathway, where 6 adolescent-specific genes and two shared genes (NMDAR and PKA) were significant for regulation by chronic nicotine (Fig. 14). By comparison, the LTP canonical pathway was not significantly overrepresented by adult-specific genes. In fact, no adult-specific genes were placed in this pathway, and two genes were shared between the age groups (not significant).

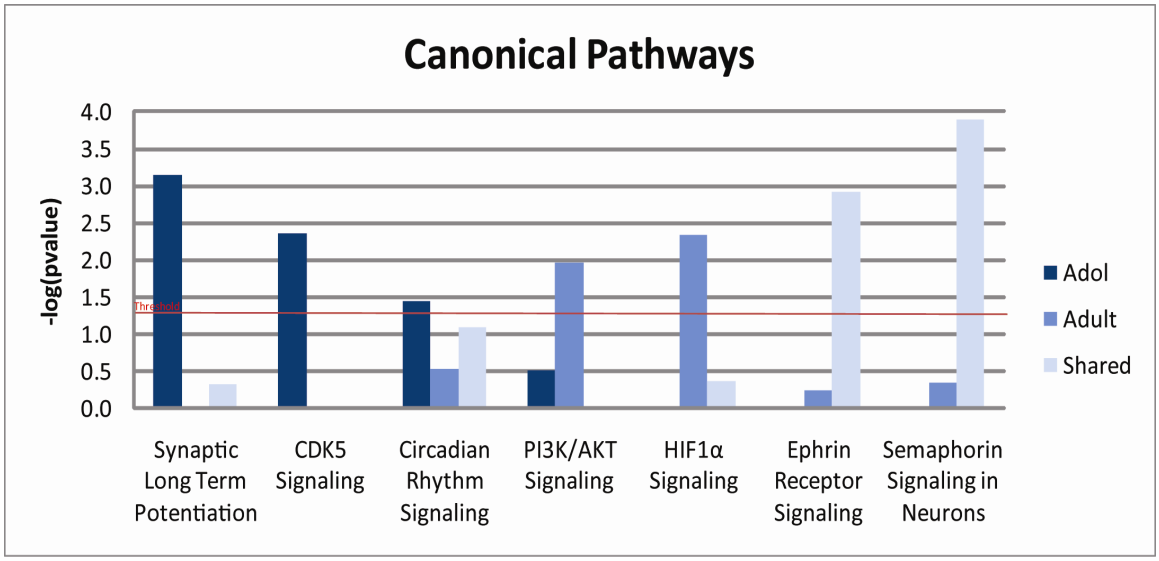

Figure 13. Canonical pathways regulated by chronic nicotine. These data were generated in IPA and are expressed as the inverse log of the p-value. The threshold line indicates $p < 0.05$ (Fisher's exact test).

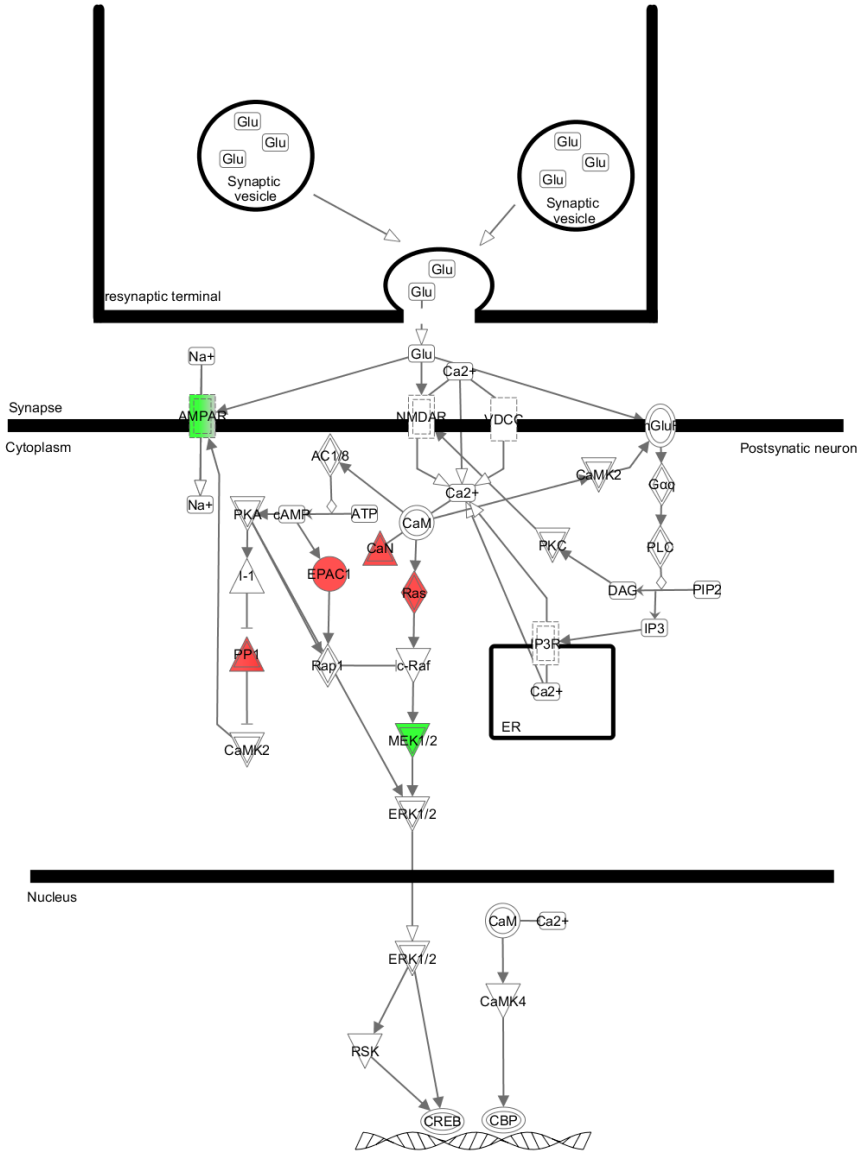

Figure 14. Synaptic long term potentiation canonical pathway in adolescent rats treated with chronic nicotine. Genes shown in red are positively correlated and genes shown in green are negatively correlated.

13. Shared biological functions and canonical pathways overrepresented in nicotine treated animals

Shared genes (transient, persistent, and late response) analyzed for biological significance (Fisher's Exact test) with IPA were similarly over-represented in the same biological functions first seen in the adolescent- and adult-specific data sets. High level biological functions identified as overrepresented by the shared genes (Fig. 11) include nervous system development and function, cell morphology, cellular development, genetic disorder, neurological disease and psychological disorders. We identified 18 schizophrenia-related genes and 19 Huntington's disease-related genes significant for nicotine regulation shared (nicotine specific/age independent) between the two age groups.

On the other hand, two overrepresented canonical pathways identified by IPA were unique to the shared data set. Top canonical pathways identified as overrepresented by the shared genes include semaphorin signaling in neurons, shown to function in synaptogenesis, axon pruning, and the density and maturation of dendritic spines (Pasterkamp & Giger, 2009), and ephrin receptor signaling, shown to function in dendritic spine morphology (Lai & Ip, 2009) (Fig. 13).

14. Nicotine regulated genes validated by QRT-PCR

Quantitative real-time RT-PCR was performed to validate microarray results of 12 adolescent-specific genes differentially regulated by nicotine (1 transient, 1 persistent and 10 late response genes; see Fig. 15). These include four genes in the LTP

canonical pathway and eight genes involved in nervous system development and function. All twelve genes were validated by RT-PCR as significant by ANOVA and post-hoc Tukey test ($P < 0.05$).

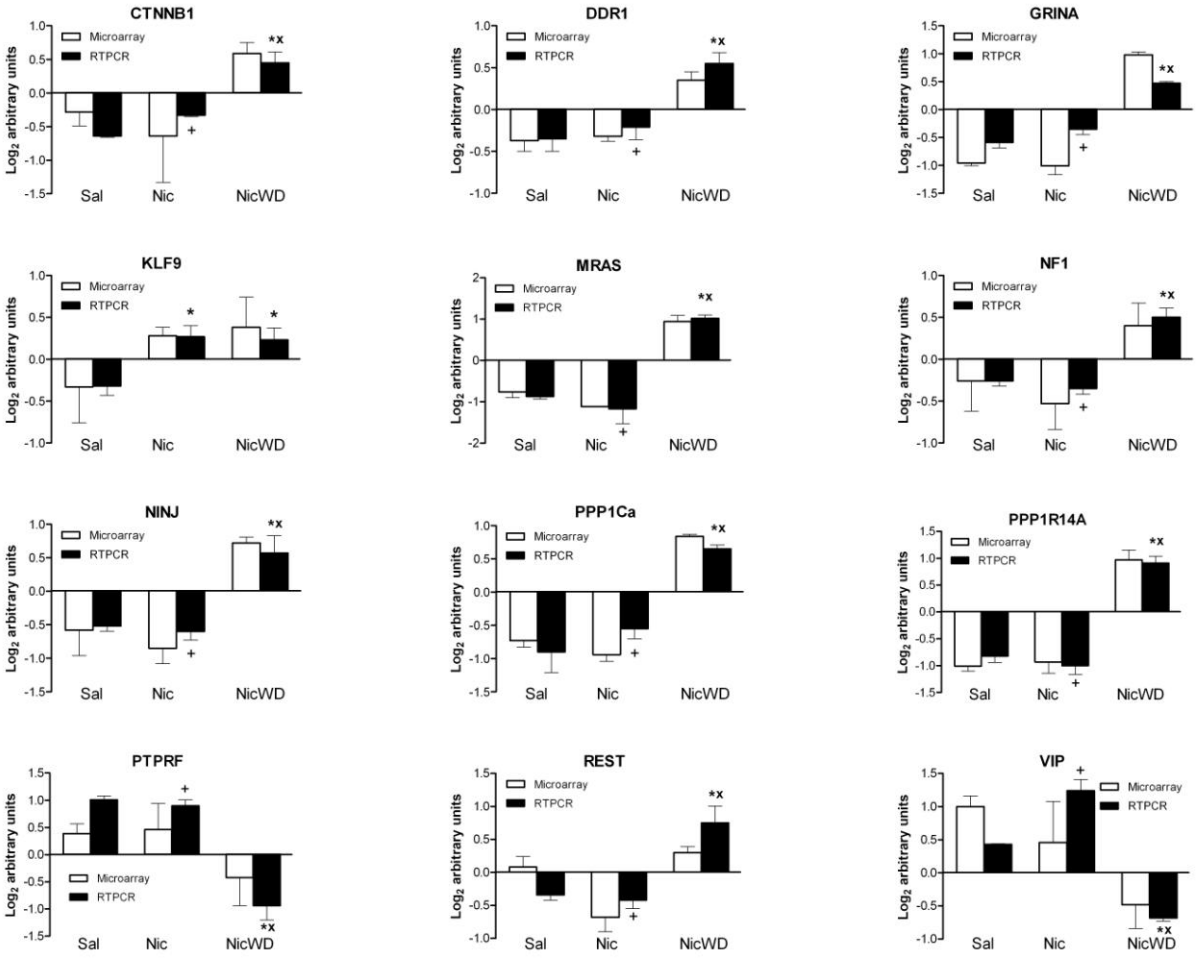

Figure 15. Quantitative real time RT-PCR validation of microarray data. Comparison of individual template matched genes measured by microarray analysis (white bars) to real time RT-PCR (black bars). Expression values for adolescents treated with saline (Sal), nicotine (Nic), or nicotine followed by 30 days withdrawal (NicWD) were compared by one-way ANOVA followed by Tukey's post-hoc test. *Significantly different from Saline; $^{+}$ significantly different from NicWD at $P<0.05$. Data are represented as the mean $\pm$ S.E.M. of 3-5 independent determinations.

Discussion

1. Developmental differences in nAChR subtype distribution

These studies are the first comprehensive characterization of the three major nAChR subtypes in adolescent and adult rat brain, and the first direct comparison of their differential response to chronic nicotine treatment in multiple brain regions. The data demonstrate that adolescent and adult rats exhibit distinct differences in the numbers of α4β2* nAChRs, and to a lesser extent α7 nAChRs, throughout the brain; however, α6* nAChRs are largely similar at both ages. Furthermore, the response of these receptors to chronic nicotine also differs by age: up-regulation of α4β2* and α7 receptors was greater in adults, but down-regulation of α6* receptors was greater in adolescents.

Developmental differences in nAChR expression have been reported in a few previous studies. Leslie and colleagues reported differences between PN20 and adult rats in expression of midbrain nAChR subunit mRNA (Azam et al., 2007). They found a gradual decline of midbrain [3H]nicotine binding after birth (although no significant differences were detected between adults and adolescents); midbrain [125I]α-Btx binding remained constant throughout development (Azam et al., 2007). [3H]cytisine binding was reported to be higher in cerebral cortex and hippocampus but not midbrain of PN45 rats compared to PN60 or PN75 rats (Trauth et al., 1999). A recent autoradiographic study in mice found that [3H]cytisine and [3H] epibatidine binding tended to peak developmentally at PN21 before declining to adult levels, while [125I]α-Btx binding peaked earlier at PN10, before also declining to adult levels (Yu et al., 2007).

Our results are generally consistent with these studies, and extend them by directly comparing the three major nAChR subtypes at both ages and in multiple brain regions.

2. Developmental differences in nAChR function

Age differences in nAChR function have also been reported. Nicotine-stimulated 86Rb efflux was found to be higher in four brain regions in PN35 rats compared to PN28 or PN63 rats (Britton et al., 2007). This method measures primarily presynaptic nAChRs, primarily α4β2* (Marks et al., 1999). These results are consistent with our findings that α4β2* binding was significantly higher in periadolescent rats (PN42) in most areas sampled from these four brain regions, suggesting that this adolescent "peak" response is the result of increased receptor expression. Nicotine-stimulated striatal dopamine release was found to be greater in PN30 compared to PN40 or adult rats (Azam et al., 2007). In vivo microdialysis studies showed that adult rats responded to an acute nicotine challenge with an increase in extracellular striatal dopamine, whereas adolescent rats did not exhibit significant increases to such a challenge (Badanich & Kirsteina, 2004). Nicotine-stimulated dopamine release from in vitro striatal preparations is mediated both by α4β2* and α6* receptors located on dopamine terminals (Champtiaux et al., 2003). Overall we found more α4β2* receptors in both dopamine cell body and terminal regions in adolescents compared to adults, but no net difference in α6* receptors. This difference in the balance between these two subtypes may contribute to an enhanced sensitivity to the rewarding effects of nicotine in adolescents.

3. α4β2* Receptors

Nicotine has long been known to cause up-regulation of its receptors following chronic administration (Schwartz & Kellar, 1983; Marks et al., 1983; Flores et al., 1992). One previous study directly compared nicotine's effects on nAChRs in adults and adolescents, using the same treatment protocol. Up-regulation of [3H]cytisine binding (largely to α4β2* nAChRs) was detected in midbrain, hippocampus and cerebral cortex of both adolescents and adults, but differences were seen in regional specificity and persistence (Trauth et al., 1999). Up-regulation varied by region in adults but was uniform in adolescents; in addition, adolescent up-regulation showed greater persistence (Trauth et al., 1999). Direct comparison with the present results is difficult given their use of homogenate binding, and the different time points employed. Similar to their findings, we saw greater increases in α4β2* binding in adult hippocampal regions compared to adolescents. We saw only modest and often non-significant increases in midbrain structures, in both age groups. As for the greater regional variability of response in adults, this is difficult to quantify; instead, we found an overall much greater responsiveness of adult α4β2* binding to up-regulation by nicotine. This may be due to differences in subunit composition between the two age groups. It has been shown that α4β2* receptors containing a α5 subunit do not increase following nicotine treatment in vivo (Mao et al., 2008).

4. α7 Receptors

Relatively less attention has been paid to the non-α4β2 nAChRs in adolescents, in part due to the paucity of selective tools. One study used homogenate binding to demonstrate modest up-regulation of [125I]α-Btx binding in the striatum and brainstem of periadolescent rats (Slotkin et al., 2004). They noted that previous studies failed to detect up-regulation in adults, and concluded that this represented a developmental difference. However, none of the studies cited used the dosing regimen employed in their study, which was the same as that used in the current study, and which we have previously shown does cause up-regulation of [125I]α-Btx binding in adult rats (Rasmussen & Perry, 2006). Clearly this subtype is less prone to up-regulation compared to α4β2* (Pauly et al., 1991). This may be a consequence of the relatively lower affinity of α7 receptors to nicotine compared to the α4β2* receptor. A similar explanation has been advanced for the resistance of α3β4* nAChRs to nicotine-induced up-regulation (Nguyen et al., 2003; Dávila-García et al., 2003).

5. α6* nAChRs

Affinity differences alone cannot explain all subtype differences in nicotine regulation: α6* nAChRs exhibit affinity for nicotine in the same nanomolar range as α4β2* nAChRs (Zoli et al., 2002), and this subtype is either not affected or is down-regulated by chronic nicotine exposure. The present results demonstrate that this subtype is unique in other ways as well. First, it does not demonstrate the same developmental arc as the α4β2* and α7 subtypes, but instead shows relatively constant levels from adolescence to adulthood. Second, its responsiveness to nicotine regulation

shows a different age-dependence than these other two subtypes: α6* nAChRs are more prone to nicotine-induced changes in adolescents than in adults. One possible explanation for these differences would be seen if the subunit composition of α6* nAChRs differed in the adolescent. It has recently shown that α6* nAChRs co-expressing the β3 subunit are resistant to nicotine-induced down-regulation compared to α6* nAChRs lacking this subunit (Perry et al., 2007).

6. Age differences in nAChR receptor response to chronic nicotine

Our studies do not differentiate between an increased number of receptors per neuron or an increase in the number of neurons expressing nAChRs. It is recognized that synaptic pruning occurs during adolescence (Spear, 2004). The widespread difference in α4β2* and α7 receptors between periadolescent and adult brains that we detected could be a result of such pruning. The age differences in responsiveness to nAChR up-regulation are more difficult to explain, in part because the mechanism(s) for such regulation remain elusive. Evidence from in vitro expression systems suggests an effect of nicotine on subunit assembly (Kuryatov et al., 2005); it is unclear how such a process might differ in adolescent brain. A generalization of the current data might be that up-regulation is greater when initial receptor levels are lower; this suggests a possible "ceiling" effect on the process. Evidence for a ceiling effect on nAChR up-regulation has not been seen with in vitro models, which often demonstrate far greater increases than seen in vivo. Furthermore, the generalization of less up-regulation with higher initial expression is far from uniform across brain regions. We recently reported

that co-expression of α5 with α4β2* nAChRs is associated with resistance to nicotine-induced up-regulation (Mao et al., 2008); as discussed above with the α6* nAChR, it is possible that adolescent α4β2* nAChRs demonstrate a different subunit composition than adults, which could affect sensitivity to up-regulation by nicotine.

Differences between subtypes in their regulation by chronic nicotine mean that the overall balance of receptor subtypes will shift with continued exposure, and presumably the response pattern to subsequent nicotine exposure. It should be pointed out that these experiments do not assess receptor functionality; because nAChRs desensitize with continued agonist stimulation, it is possible that the rate or extent of desensitization, or recovery from desensitization, differs in adult and adolescent animals. We have previously shown that the increased numbers of α4β2-like receptors following identical chronic nicotine exposure in adult rats represent functional receptors, as assayed by 86Rb efflux (Nguyen et al., 2003), and that the decreased numbers of α6* receptors in striatum were accompanied by a like decrease in αCtxMII-sensitive nicotine stimulated dopamine release from striatal synaptosomes (Perry et al., 2007).

Collectively the binding data shows that adolescent rats have a distinct pattern of nAChR expression, and respond differently to chronic nicotine exposure, compared to adult rats. A different pattern of CNS nicotinic receptor expression may play a role in the initiation of smoking among adolescents. Furthermore, the distinct pattern of responses of nAChR subtypes to nicotine during adolescence may contribute to the

higher daily consumption and decreased probability of cessation observed in smokers who initiate tobacco use during adolescence.

7. Nicotine produces age-specific patterns of gene regulation

A number of studies have been conducted examining the gene expression profiles of animals exposed to drugs of abuse in an attempt to identify the genetic pathways involved in the etiology of addiction. Thus far, few studies have examined the differences in the response of adolescent and adult animals. Epidemiological data clearly suggest that adolescents exposed to drugs of abuse have unique responses which persist into adulthood. We have conducted whole genome mRNA analysis of the VTA from adolescent and adult SD rats treated with chronic nicotine and find that adolescent rats also show unique genomic responses that persist into adulthood.

A two-way ANOVA identifying age specific genes revealed that adolescent rats have a strikingly distinct response to nicotine's genetic regulatory effects relative to adults. Adolescent animals had only half as many age-specific genes affected transiently, whereas they had twice as many age-specific genes regulated persistently, and five times as many showing delayed regulation relative to adult animals. This suggests the developing adolescent brain may be significantly more sensitive to long-lasting effects of nicotine exposure at the transcriptional level. This sensitivity may relate to documented sensitivities of adolescents to the addictive effects of nicotine (and other drugs of abuse). Taken together, this suggests the age of onset of tobacco use significantly affects the response to exposure. Further, chronic nicotine exposure

causes downstream effects that are seen long after cessation of treatment. This may cause changes in the developing adolescent brain which persist into adulthood and augment response to later drug exposure.

Over-represented classes of genes specific to adolescent chronic nicotine exposure included those associated with genetic and psychological disorders, developmental disorders, and nervous system development and function. Network analysis of genes from the latter category revealed that these adolescent-specific nicotine-regulated genes form extensive networks in adolescent VTA, but that this is not the case for adult-specific genes in the VTA. A number of genes unique to adolescents were found to regulate neurite structure. Groups of genes were identified that regulate development of neurites (11 genes), neurite morphogenesis (7 genes), growth of neurites (15 genes), length (4 genes) and branching of neurites (5 genes). These genes are of particular interest because of their possible roles in structural and behavioral plasticity. Pathway analysis showed several canonical pathways over-represented by adolescent-specific genes in adolescent VTA, notably long-term potentiation, circadian rhythm signaling and CDK5 signaling. Animal studies have found that the circadian genes are important regulators of drug responsiveness, sensitization, and reward (Falcón & McClung, 2009). CDK5 has been shown to modulate dopamine signaling in neurons (Bibb et al., 1999). Pathways over-represented by adult-specific genes included protein ubiquitination, PI3K/AKT signaling and HIF1α signaling. PI3K/AKT signaling is activated by other drugs of abuse, and is known to function in neuronal branching, dendritic spine density, and cell body size (Russo et al., 2009).

8. Over-represented biological functions

Two recent meta-analysis examined separately generated lists of drug addiction related genes chosen from literature reviews for over-represented high-level biological functions in order to identify biological processes involved in drug addiction. Sun and Zhoa (2010) conducted bioinformatic analysis of 62 addiction related genes and identified 13 high-level biological pathways over-represented in the gene list. Wang and Li (2010) examined three gene lists (smoking initiation, nicotine dependence, and smoking cessation) for over-represented biological processes. They identified 9 pathways over-represented in smoking initiation, 21 pathways over-represented in nicotine dependence, and 13 pathways over-represented in smoking cessation. Bioinformatic analysis of our age-specific and shared genes revealed significant overlap between these data and our own. Of the 13 over-represented pathways identified by Sun and Zhao, nine are also over-represented in our data. These pathways include G-protein coupled receptor signaling, calcium signaling, cAMP-mediated signaling, glutamate receptor signaling, neuropathic pain in the dorsal horn neurons, dopamine receptor signaling, LTP, circadian rhythm signaling, and long term depression (LTD). Comparing our data to Wang and Li, seven of the 9 over-represented smoking initiation related pathways are also over-represented in our data, including calcium signaling, dopamine receptor signaling, cAMP-mediated signaling, G-protein coupled receptor mediated signaling, tight junction signaling, IL-8 signaling, and integrin signaling. Eleven of the 13 smoking cessation related pathways are also over-represented in our data.

These pathways include glutamate receptor signaling, circadian rhythm signaling, ALS signaling, LTP, LTD, neurotrophin/TRK signaling, and several pathways also over-represented in smoking initiation. There is less agreement when comparing our data to results from the nicotine dependence gene set. Only eight of the 21 nicotine dependence pathways are also over-represented in our data. Interestingly, the majority of the pathways that do not reach significance in our data set are metabolism pathways (8 of 11 unconfirmed pathways). The confirmed pathways include dopamine receptor signaling, cAMP-mediated signaling, G-protein coupled receptor mediated signaling, calcium signaling, GABA receptor signaling, neurotrophin/TRK signaling, LPS/IL1 signaling, PXR/RXR signaling, LTP, and regulation of actin cytoskeleton. These data suggest further study of addiction should focus on these high-level biological pathways. Greater comprehension of the affect of drug exposure on these pathways and the interaction networks between these pathways will further our understanding of the etiology of substance addiction.

9. Nervous system development and function

Drugs of abuse are known to produce widespread effects on neuronal structure and function. This is hypothesized to be the basis for the long lasting changes in behavior seen in addiction. Long-term changes that occur within the brain's reward circuitry are central to reward related behaviors and drug response. In particular, the changes in dopaminergic neurons of the VTA and NAcc are thought to alter reward

responses leading to tolerance, increased intake, reward dysfunction, and addiction (Russo et al., 2009).

Changes in spine morphology are critical for synaptic function (Sorra & Harris, 2000; Hering & Sheng, 2001; Yuste & Bonhoeffer, 2001). For instance, larger spines support stronger synaptic transmission (El-Husseini et al., 2000; Murthy et al., 2001). Spine morphology also plays an important role in synaptic plasticity. The autonomous space created by the spine neck facilitates activity-triggered calcium increases (Wang et al., 2000; Sabatini et al., 2002), which activates calcium-activated kinases and phosphatases that underlies synaptic plasticity (Morishita et al., 2001; Thiels and Klann, 2001; Lisman et al., 2002). Chronic administration of cocaine and amphetamine produce increased spine density in the NAcc (Robinson & Kolb, 1997, 1999a). Similarly, chronic nicotine exposure increases spine density within the NAcc and medial prefrontal cortex (Brown & Kolb, 2001; Robinson & Kolb, 2004). Opioids have the opposite effect: morphine decreases spine density and dendritic branching in the accumbens shell and medial prefrontal cortex (Robinson & Kolb, 1999b; Robinson et al., 2002; Robinson & Kolb, 2004). These structural changes are seen long after cessation of drug treatment, up to 3.5 months (Kolb et al., 2003; Robinson & Kolb, 2004).

10. Circadian rhythms

Drosophila studies first showed that mutations in the circadian rhythm genes Per, Clock, Cycle, or Doubletime had an effect (fail to sensitize after repeated exposure) on cocaine response (Andretic et al., 1999). Several subsequent studies have shown

that cocaine can induce or repress the expression of circadian rhythm genes in several brain regions (Yuferov et al., 2003; McClung & Nestler, 2003; Uz et al., 2005). Mice lacking a functional mPer1 gene were demonstrated to have no CPP response, and failed to sensitize to cocaine (Abarca et al., 2002). Mice with a mutant Clock gene, however, showed robust cocaine sensitization, increased cocaine preference, and increased reward as measured by intracranial self-stimulation following cocaine treatment (McClung et al., 2005; Roybal et al., 2007). A recent microarray study identified 29 circadian rhythm genes differentially regulated in animals self-administering cocaine (Lynch et al., 2008). Studies have also suggested that circadian rhythm genes play a role in alcohol (Spanagel et al., 2005) and morphine (Liu et al., 2005) response. These data and our own suggest that drugs of abuse alter expression of circadian rhythm genes. Other studies show altering circadian rhythm gene expression alters drug sensitivity and reward. This suggests a direct role for circadian rhythm genes in substance abuse. Further study of this area may prove useful in understanding the differential response to drugs of abuse, and identifying therapeutic targets for the treatment of various forms of drug addiction.

11. Long term potentiation (LTP)

There is a large body of data supporting the involvement of LTP in drug addiction. In fact, addiction has been described as “a pathological usurpation of learning and memory” (Hyman, 2005). Behavioral sensitization, conditioned locomotor response, and CPP are drug-associated phenomena that are context- and NMDA-

dependent, suggesting the involvement of LTP (Hyman & Malenka, 2001). Evidence suggests that drug-induced synaptic plasticity is mediated by a process similar to long term potentiation (Ungless et al., 2001; Saal et al., 2003). A single dose of a drug may be enough to change the AMPA/NMDA receptor balance that blocks LTP induction (Ungless et al., 2001; Saal et al., 2003). Psychostimulants may facilitate synaptic potentiation by interfering with long term depression (LTD) in the same cells (Jones et al., 2000; Gutlerner et al., 2002). Neuronal nicotinic receptors are known to play a role in cognition; recent studies have detailed the effects of nicotine on learning and LTP (Gold et al., 2009; Tang & Dani, 2009).

Synaptic plasticity in the VTA has been implicated in the acquisition of nicotine dependence (Dani et al., 2001). It has been demonstrated that a single dose of nicotine is sufficient to induce synaptic plasticity in VTA DA neurons (Gray et al., 1996; Mansvelder et al., 2000; Saal et al., 2003; Pidoplichko et al., 2004). These synaptic alterations correlate with behavioral sensitization (Borgland et al., 2004) and self-administration (Chen et al., 2008) in animal studies. Prolonged nicotine exposure causes desensitization of β_2-containing nicotinic receptors, reducing GABAergic inhibitory tone and increasing DA neuron excitability and the probability of glutamatergic LTP (Dani et al., 2001).

While epidemiological data clearly show that adolescents are more vulnerable to drugs of abuse, and a number of studies have examined the effects of addictive drugs in adolescent rodent models, little is known about the developmental factors affecting changes in plasticity caused by nicotine. The hippocampus is vital for encoding and

recalling episodic memory (Squire et al., 2004). It is now clear that DA affects LTP, and that the hippocampus receives significant DA innervation from the VTA (Gasbarri et al., 1997). DA cells increase burst firing in response to unexpected reward and reduce burst firing when expected reward is omitted (Schultz & Dickinson, 2000). Both conscious and unconscious memory mechanisms contribute to the rewarding effects of nicotine and other drugs of abuse (Kauer & Malenka, 2007; Kelley, 2004; Dani & Montague, 2007). Nicotine increases neuronal excitability and strengthens synapses in several brain regions (Kauer & Malenka, 2007; Mansvelder & McGehee, 2002). Recent evidence suggests rather than directly encoding reward, DA signals contribute to associative learning in response to behaviorally important cues (Schultz, 2007). Nicotine-induced DA signaling in hippocampus has been shown to be necessary for LTP induction and nicotine reinforcement as measured by CPP (Tang & Dani, 2009).

Exposure to nicotine and other drugs of abuse induces long term changes similar to LTP in the motivational/reward pathway (Jones & Bonci, 2005; Kauer & Malenka, 2007). Identifying the mechanism by which nicotine induces conscious and unconscious memory is important for understanding addiction. Further, the differential response to chronic nicotine exposure seen between our adolescent and adult animals may play a role in smoking acquisition and the disparate outcomes observed between adolescent and adult smokers.

We have demonstrated striking differences in gene expression response of adolescent and adult SD rats to chronic nicotine exposure. VTA gene expression profiles reveal a response pattern suggesting adolescents are more severely affected in the long

term relative to adult animals. Further, chronic nicotine was shown to affect genes associated with neuronal structure and function, LTP, circadian rhythm, and a number of psychological disorder related pathways more robustly in our adolescent animals. Analysis of the significant genes shared between age groups revealed convergence in some pathways (nervous system and development, circadian rhythms) relative to age specific genes. These effects were much more robust, however, in our adolescent animals. Our data suggest that adolescent rats have a greater sensitivity to chronic nicotine's effects and show a prolonged response in a number of genetic pathways lasting well into adulthood. Further study of these areas may provide promising targets for the treatment of drug abuse.

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