Universidade do Estado do Rio de Janeiro
Centro Biomédico
Faculdade de Ciências Médicas
Pós-graduação em Fisiopatologia Clínica e Experimental

Anderson Ribeiro Carvalho

CURSO TEMPORAL DOS EFEITOS DA EXPOSIÇÃO COMBINADA DE NICOTINA E ETANOL NO SISTEMA COLINÉRGICO CEREBRAL DURANTE ADOLESCÊNCIA

RIO DE JANEIRO
2009

Livros Grátis

http://www.livrosgratis.com.br

Milhares de livros grátis para download.

Anderson Ribeiro Carvalho

CURSO TEMPORAL DOS EFEITOS DA EXPOSIÇÃO COMBINADA DE NICOTINA E ETANOL NO SISTEMA COLINÉRGICO CEREBRAL DURANTE ADOLESCÊNCIA

Tese apresentada ao Curso de Pós-Graduação em Fisiopatologia Clínica e Experimental da Universidade do Estado do Rio de Janeiro para a obtenção do grau de Doutor em Ciências.

Orientadora: Profª Dra. Yael de Abreu Villaça

Co-orientador: Profº Dr. Cláudio Carneiro Filgueiras

RIO DE JANEIRO
2009

CATALOGAÇÃO NA FONTE
UERJ/REDE SIRIUS/BIBLIOTECA CB-A

C331 Carvalho, Anderson Ribeiro.
Curso temporal dos efeitos da exposição combinada de nicotina e etanol no sistema colinérgico cerebral durante adolescência / Anderson Ribeiro Carvalho. - 2009.
cxxiii, 1016f. : il.

Orientador : Yael de Abreu-Villaça.
Co-orientador : Cláudio Carneiro Filgueiras.
Tese (Doutorado) – Universidade do Estado do Rio de Janeiro, Faculdade de Ciências Médicas.Pós-Graduação em Fisiopatologia Clínica e Experimental.
Bibliografia: f. 84-1016.

1. Adolescência - Alcool - Teses. 2. Nicotina -Teses. 3. Etanol - Teses. 4. Memória - Teses. 5. Aprendizagem - Teses. 6. Ansiedade - Teses.7. Manifestações neurocomportamentais - Teses. 8. Hiperfagia - Teses.I. Abreu-Villaça, Yael de. II. Filgueiras, Cláudio Carneiro. III. Universidade do Estado do Rio de Janeiro.Faculdade de Ciências Médicas. IV. Título.

CDU 616-053.7:613.81

Anderson Ribeiro Carvalho

CURSO TEMPORAL DOS EFEITOS DA EXPOSIÇÃO COMBINADA DE NICOTINA E ETANOL NO SISTEMA COLINÉRGICO CEREBRAL DURANTE ADOLESCÊNCIA

Orientadora: Profª Dra. Yael de Abreu Villaça
Co-orientador: Profº Dr. Cláudio Carneiro Filgueiras

Aprovada em ________ de __________________ de 2009 pela banca examinadora:

Prof. Dr.: ____

(Profª Yael Abreu Villaça - UERJ)

Prof. Dr.: ____

(Profª. Penha Cristina Barradas Daltro Santos - UERJ)

Prof. Dr.: ____

(Profª. Thereza Christina Barja Fidalgo - UERJ)

Prof. Dr.: ____

(Profª. Patrícia Franca Gardino - UFRJ)

Prof. Dr.: ____

(Profª. Tania Marcourakis - USP)

RIO DE JANEIRO
2009

Dedico este trabalho a toda minha família, especialmente aos meus avós.

AGRADECIMENTOS

- A minha amada esposa Renata. Sua companhia, incentivo, compreensão e dedicação foram imprescindíveis para vencer essa grande etapa da minha vida. Não dá para traduzir em palavras a sua importância em minha vida! Sem você nada seria possível!

- A minha orientadora Yael Abreu Villaça, por sua atenção, apoio, confiança. Você é um exemplo de dedicação e me faz acreditar que o trabalho ainda pode dignificar o homem.

- Ao amigo Profº Cláudio Carneiro Filgueiras. É imensurável o que pude aprender contigo durante esses quase oito anos de convívio. Atualmente, onde o tempo é um bem precioso, você ensina o quanto à reflexão e a ética são importante na vida de um pesquisador. Como você mesmo disse: "Nós somos um grupo forte porque acima de tudo a gente se gosta". E é por isso que sempre vale a pena acordar cedo e ir pro laboratório nos finais de semana e feriados.

- Ao amigo Profº Alex Christian Manhães pelo apoio e incentivo dado em todos os momentos da minha jornada científica.

- A minha amiga Carla Soares, que contribui enormemente para a realização deste trabalho. Você é um exemplo de pessoa! O mundo seria muito melhor se todos tivessem metade da sua ética, compromisso e responsabilidade.

- Aos grandes amigos "das antigas" do laboratório: Fernanda, Ulisses e Dani.

- A todos os outros colegas de laboratório, pelo agradável convívio que tivemos ao longo de todos esses meses, em especial pelos amigos: Andréia, André, Julianas, Mabel e Aninhas.

- Aos camundongos.

- A Colina, ao judô, ao jiujitsu e é claro ao Rock'n Roll.

RESUMO

O etanol e a nicotina são as drogas mais comumente usadas no mundo. Como claramente indicado por estudos epidemiológicos, existe uma forte associação entre o tabagismo e o consumo de etanol principalmente durante o período da adolescência. Entretanto, existem poucos estudos em neurobiologia básica que avaliem o efeito da exposição combinada de nicotina e etanol durante o período da adolescência. Considerando que a nicotina é um agonista do receptor colinérgico nicotínico (nAChR) e que tem sido demonstrado que o etanol interage com os nAChRs, o presente trabalho tem como foco o estudo dos efeitos da exposição à nicotina e/ou ao etanol no sistema colinérgico durante a adolescência. Do 30º ao 45º dia pós-natal (PN) camundongos da cepa C57BL/6 foram expostos à nicotina (NIC) e/ou etanol (ETOH). Quatro grupos foram analisados: 1) exposição concomitante (NIC+ETOH) à solução de nicotina (50µg/ml) e etanol (25%, 2g/kg i.p. em dias alternados), 2) exposição a NIC, 3) exposição ao ETOH, 4) exposição ao veículo. Foram quantificadas a expressão/afinidade aos nAChRs ($\alpha4\beta2$), a atividade da colina acetiltransferase (ChAT) e, a expressão/afinidade do [^{3}H]hemicolinium-3 (HC-3) ao transportador de alta afinidade pré-sinaptico de colina, ao final da exposição (PN45), após curto (PN50) e longo período de retirada (PN75). Ao final da exposição, o grupo NIC+ETOH apresentou *upregulation* de nAChRs, refletindo simples somação dos efeitos da NIC e ETOH no córtex cerebral e sinergismo no mesencéfalo. A *upregulation* devido à exposição combinada foi mantida mesmo após alguns dias de retirada das drogas. Um mês após o término da exposição, os valores foram semelhantes aos obtidos para os animais veículo. Em PN45, machos NIC apresentaram aumento da ChAT no córtex cerebral, mas o ETOH foi capaz de reverter este efeito. Ao contrário, fêmeas NIC apresentaram diminuição da ChAT. No mesencéfalo, somente ETOH promoveu aumento da ChAT. Já em PN50, o grupo NIC apresentou aumento na ChAT que foi revertido na retirada combinada de NIC+ETOH. Em PN75, o grupo NIC+ETOH apresentou redução da ChAT. Em relação ao HC-3, não houve alterações ao final da exposição. ETOH e NIC+ETOH apresentaram redução da marcação alguns dias após a retirada das drogas, a qual persistiu até PN75. O presente trabalho fornece evidências experimentais que indicam que nicotina e etanol interagem durante a adolescência resultando em alterações nos sistema colinérgico durante a exposição e durante a retirada das drogas.

ABSTRACT

Nicotine and ethanol are the most commonly consumed drugs. As clearly indicated by epidemiological studies, there is a close interrelationship between smoking and alcohol consumption manly during adolescence period. However, there are few studies on the basic neurobiology of the effects of the combined nicotine and ethanol exposure in the adolescent brain. Since nicotine is a cholinergic agonist and it has been shown that ethanol interferes with nicotinic acetylcholine receptors (nAChR), the current proposal will focus on the cholinergic effects of nicotine and/or ethanol treatment during adolescence. From the 30th to the 45th postnatal day (PN), C57BL/6 mice were exposed to nicotine free base (NIC) and/or ethanol (ETOH). Four groups were analyzed: 1) concomitant (NIC+ETOH) exposure of nicotine (50 μg/ml) and ethanol (25%, 2 g/kg i.p. every other day); 2) NIC exposure; 3) ETOH exposure; 4) vehicle. We assessed nAChR ($\alpha4\beta2$) binding, choline acetyltransferase (ChAT) activity and [3H]hemicholinium-3 (HC-3) binding to the high affinity presynaptic choline transporter at the end of exposure period (PN45), at short (PN50) and long term (PN75) withdrawal. At the end of exposure period, NIC+ETOH elicited a pronounced *upregulation* which reflect simple additivity of the effects of nicotine and ethanol in the cerebral cortex and synergism in the midbrain. On PN45, male NIC mice presented an increase in ChAT in the cerebral cortex. However, ETOH reversed this effect. In contrast, female NIC mice presented decreased ChAT activity. In the midbrain, ETOH increased ChAT. On PN50, NIC mice presented an increase in ChAT activity that was reversed by ETOH withdrawal. In addition, NIC+ETOH long term withdrawal elicited a decrease in ChAT activity. Regarding HC-3, binding was not affected on PN45. ETOH and NIC+ETOH withdrawal promoted a decrease at short and long-term withdrawal. These results provide experimental evidences that nicotine and ethanol during adolescence interact resulting in cholinergic system alterations during exposure and withdrawal.

LISTA DE TABELAS

Pág.

ARTIGO 1: Tabela 1 – Médias do consumo de líquido, peso corporal e peso das regiões cerebrais. 40

ARTIGO 1: Tabela 2 – Médias dos biomarcadores colinérgicos encontradas no grupo controle. 40

ARTIGO 2: Tabela 1 – Médias do peso das regiões cerebrais. 55

Tabela 1- Resumo dos resultados. 68

LISTA DE ILUSTRAÇÕES

Pág.

Figura 1 - Linha temporal do modelo experimental utilizado no presente estudo. 36

ARTIGO 1: Figura 1 – Efeitos da exposição a nicotina e/ou etanol sobre os nAChRs em PN45. 40

ARTIGO 1: Figura 2 – Efeitos da exposição a nicotina e/ou etanol sobre a ChAT em PN45. 41

ARTIGO 1: Figura 3 – Efeitos da exposição a nicotina e/ou etanol sobre o HC-3 em PN45. 42

ARTIGO 2: Figura 1 – Efeitos da exposição a nicotina e/ou etanol sobre o consumo de líquidos e peso corporal. 55

ARTIGO 2: Figura 2 – Efeitos da exposição a nicotina e/ou etanol sobre os nAChRs após curto (PN50) e longo (PN75) período de retirada. 56

ARTIGO 2: Figura 3 – Efeitos da exposição a nicotina e/ou etanol sobre a ChAT após curto (PN50) e longo (PN75) período de retirada. 57

ARTIGO 2: Figura 4 – Efeitos da exposição a nicotina e/ou etanol sobre o HC-3 após curto (PN50) e longo (PN75) período de retirada. 58

LISTA DE SIGLAS E ABREVIATURAS

5-HT - 5-hidroxitriptamina

ANOVA - Análise de Variância.

ChAT – colina acetiltransferase

E.P. – Erro padrão da média.

ETOH - grupo de animais expostos ao etanol

F - razão de Fischer.

FPLSD - *Fisher Protected Least Significant Difference*

g.l. – Graus de liberdade.

GABA - Ácido gama-aminobutírico

HC-3 - [3H]hemicholinium-3

i.p. - intraperitoneal

nAChR - receptor nicotínico da acetilcolina

NIC – grupo de animais expostos à nicotina

NIC+ETOH – grupo de animais expostos simultaneamente à nicotina e ao etanol

NMDA - N-metil D-aspartato

P – Valor de prova.

PN – dia de vida pós-natal

rANOVA – Análise de Variância com medidas repetidas

t – t de Student.

VEH – grupo controle

SUMÁRIO

	Página
1. INTRODUÇÃO	13
1.1. Nicotina	13
1.1.1. Metabolismo	14
1.1.2. Efeitos neuroquímicos	15
1.2. Etanol	19
1.2.1. Metabolismo	20
1.2.2. Efeitos neuroquímicos	21
1.3. Co-uso e co-abuso	24
1.3.1. Metabolismo	25
1.3.2. Neuroquímica do co-uso e co-abuso	26
1.4. Adolescência como período de vulnerabilidade	29
1.5. Estudos animais sobre a interação nicotina e etanol durante a adolescência	32
2. OBJETIVOS	34
3. RESUMO DA METODOLOGIA (DESENHO EXPERIMENTAL)	35
4. ARTIGO 1	37
5. ARTIGO 2	51
6. DISCUSSÃO	65
6.1. Resumo dos resultados	65
6.1.1. Durante o período de exposição	65
6.1.2. Durante o período de retirada	66
6.2. Considerações metodológicas	68
6.2.1. Camundongos C57BL/6	68
6.2.2. Concentração de etanol no sangue e níveis de cotinina	69
6.2.3. Significado e interpretação dos marcadores biológicos	71
6.2.4. Alterações na ingesta e peso	73
6.3. Efeitos sobre o sistema colinérgico	73
6.3.1. Efeitos sobre nAChRs	73

6.3.2. Efeitos sobre a ChAT e HC-3 77
6.4. Possibilidades de outros sítios de interação entre nicotina e etanol 82

7. CONCLUSÕES 83

8. REFERENCIAS BIBLIOGRÁFICAS 84

1. INTRODUÇÃO

1.1. Nicotina

"O CIGARRO NÃO DEVERIA SER CONSIDERADO COMO UM PRODUTO, MAS SIM COMO UM INVÓLUCRO. O PRODUTO É A NICOTINA. CONSIDERE O MAÇO DE CIGARROS COMO UM RECIPIENTE PARA O FORNECIMENTO DIÁRIO DE NICOTINA".
PHILLIP MORRIS, 1972

Existe em torno de 1-2 bilhões de fumantes no mundo, sendo que metade destes irão morrer devido a doenças relacionadas com o tabagismo. O total de mortes devido ao uso do tabaco atingiu o número de 4,9 milhões por ano, o que corresponde a mais de 10 mil mortes por dia (WHO, 2003). A prevalência pode variar entre os países; particularmente, é estimado que nas grandes capitais brasileiras em torno de 20% dos adultos são fumantes (INCA, 2004).

A nicotina é um alcalóide vegetal e hoje é considerada o componente que estabelece e mantém a dependência no tabagismo (Di Chiara, 2000; Stolerman e Shoaib, 1991). Assim, acredita-se que o tabagismo seja a forma pela qual o indivíduo consegue consumir a nicotina. Apesar dos efeitos do tabaco em relação à saúde e da capacidade viciante da nicotina serem bem conhecidos, consome-se, anualmente no mundo, a grande quantidade de 73 mil toneladas de nicotina (WHO, 2004).

Vale ressaltar que a maioria dos fumantes inicia o hábito de fumar durante a adolescência (Mansvelder e McGehee, 2002), período no qual o indivíduo parece ser mais susceptível ao vício pela nicotina. Estudos mostram que o consumo de cigarros diários é maior em indivíduos que iniciam o hábito na adolescência quando comparados com indivíduos que iniciam o hábito de fumar na idade adulta (Chen e Millar, 1998; Nelson et al., 1995; Pierce e Gilpin, 1996). Adicionalmente, adolescentes já expressam sintomas de dependência à nicotina após o consumo de apenas alguns cigarros (Di Franza et al., 2000).

1.1.1. Metabolismo

Análises em série das concentrações de nicotina no sangue de tabagistas revelam sua rápida elevação após a tragada, atingindo o pico máximo ao cabo de 5 a 10 minutos. A rápida taxa de absorção da nicotina e as grandes quantidades de nicotina que chegam ao cérebro pelo tabagismo são dois fatores cruciais na promoção e manutenção do vício. A absorção da nicotina pelo organismo é em média 1,0 mg por cigarro, variando de 0,34 mg a 1,56 mg (Benowitz, 1998). A absorção da nicotina pode ser influenciada por sua concentração presente no tabaco, pela maneira de fumar e pela profundidade das tragadas. Depois da absorção, a nicotina se distribui facilmente pelo organismo através da corrente sanguínea. Baseado em autopsias de fumantes, as regiões corporais que possuem maior afinidade pela nicotina são o fígado, rins, baço e principalmente o sistema nervoso central (Urakawa et al., 1994). A meia vida da nicotina é cerca de duas horas em seres humanos. Somando-se os valores das meias-vidas do consumo de tabaco subseqüentes, pode-se prever que a nicotina se acumula, num fumante regular, a níveis significantes sanguíneos por pelo menos 6 a 8 horas depois de cessado de fumar (Hukkanen et al., 2005).

Nicotina é extensivamente metabolizada em um grande número de metabólitos pelo fígado. Na maioria dos mamíferos, incluindo roedores e humanos, o principal metabólito da nicotina é a cotinina (Hukkanen et al., 2005). Em humanos cerca de 70 a 80% da nicotina é convertida em cotinina (Benowitz e Jacob, 1994). A cotinina, por possuir uma meia-vida plasmática mais longa, tem sido utilizada como marcador da exposição ao tabagismo. A enzima CYP2A6 do citocromo P450 tem sido demonstrada como a enzima que é primariamente responsável pelo metabolismo da nicotina em humanos (Hukkanen et al., 2005). Vários polimorfismos tem sido descritos para essa enzima (Hukkanen et al., 2005; Oscarson, 2001). Além da variabilidade genética, vários outros fatores geram uma grande

variação entre indivíduos no metabolismo da nicotina (Benowitz et al., 1982). Entre esses fatores destacam-se: dieta, idade, gênero e uso de medicações.

1.1.2. Efeitos neuroquímicos

Está consolidado na literatura que os principais efeitos comportamentais da nicotina são mediados pela ativação dos receptores nicotínicos de acetilcolina (nAChRs) (Dani e De Biasi, 2001; Wonnacott et al., 2005). Os nAChRs são receptores ionotrópicos pentaméricos e estão localizados em sítios pré e pós sinápticos no sistema nervoso central e periférico (Leonard e Bertrand, 2001; Sargent, 2000). Existe uma grande variedade de subtipos de nAChRs que surge das possibilidades de combinações de cada uma das suas subunidades. Em mamíferos, já foram clonadas 8 subunidades α (α2 a α7, α9 e α10) e 3 subunidades β (β2 a β4) (Elgoyhen et al., 1994, 2001; Lindstrom et al., 1996; McGehee e Role, 1995; Sargent, 1993; Tassonyi et al., 2002). A diversidade dos subtipos de nAChR, sua localização (pré ou pós sinaptica) e sua distribuição cerebral contribuem para a variedade de mecanismos que estes receptores participam no sistema nervoso central. A ativação dos receptores pré sinapticos de modo geral promove a liberação de outros neurotransmissores, enquanto que, a ativação de receptores pós sinapticos promove excitação neuronal, como também, a modulação da atividade enzimática (Aramakis e Metherate, 1998; Dani et al., 2000). Desta forma, nAChRs participam em aspectos fundamentais na plasticidade sinaptica envolvida em muitos processos fisiológicos e patológicos incluindo o desenvolvimento neuronal, aprendizado e memória, e respostas de reforço induzidas por drogas de abuso (Gopalakrishnan et al., 1997; Jang et al., 2002; Levin e Simon, 1998; Salamone e Zhou, 2000). Há evidências de que nAChRs também estão envolvidos em processos como ansiedade (File et al., 2000; Ross et

al., 2000), controle do sono (Domino e Yamamoto, 1965; Salin-Pascual et al., 1999), antinocicepção (Marubio et al., 1999) e funções do sistema nervoso autônomo (De Biasi, 2002; Xu et al., 1999a, b).

Devido ao fato de a nicotina regular a liberação de uma grande variedade de neurotransmissores (Wonnacott, 1997), o padrão de funcionamento de vários outros sistemas de neurotransmissores podem ser afetados. O sistema dopaminérgico é um dos mais afetados pela estimulação dos nAChRs pela nicotina. Neste sentido, é muito bem conhecido que a nicotina induz liberação de dopamina em todas as grandes vias dopaminérgicas, incluindo a via nigroestriatal e a via mesolímbica. Particularmente, a via mesolímbica tem sido o foco de estudos sobre os efeitos da nicotina sobre o sistema dopaminérgico, pois é uma via fundamental para a geração dos efeitos de reforço e recompensa, fundamentais na geração do vício pela nicotina, como também, por outras drogas de abuso (Balfour et al., 1998; Janhunen e Ahtee, et al., 2007). Esta via tem origem na área do tegumento ventral do mesencéfalo e projeta para o núcleo acumbente, sistema límbico e córtex orbitofrontal. Além do sistema dopaminérgico, tem sido descritas alterações nos sistemas setotoninérgicos, noradrenérgicos e GABAérgicos (Slotkin e Seidler, 2009; Xiao et al., 2009; Zhao et al., 2007).

Apesar do grande número de estudos, os mecanismos moleculares envolvidos no processo de geração do vício são pouco conhecidos, e dependendo do sistema estudado, a droga pode promover respostas complexas, variadas e difíceis de interpretar. De forma geral, a nicotina promove uma rápida dessensibilização dos nAChRs, que promoverá, em conseqüência, uma aumento da afinidade por agonistas e/ou aumento da expressão dos nAChRs (*upregulation*). Essa *upregulation* está em contraste com os mecanismos envolvendo outros sistemas de neurotransmissores que normalmente apresentam *downregulation* quando expostos cronicamente a seus agonistas. Sugere-se amplamente na literatura que esses efeitos

promovidos pela nicotina têm papel importante na geração dos seus mecanismos de vício (Buisson e Bertrand, 2001, 2002; Dani e De Biasi, 2001; Quick e Lester, 2002).

A dessensibilização é uma característica geral de canais do tipo portão dependente de neurotransmissores, e pode ser definida como uma diminuição ou perda de resposta biológica destes canais em resposta a uma estimulação prolongada ou repetitiva. Este processo resulta da capacidade da nicotina em estabilizar a conformação inativa dos nAChRs. Desta forma, após a exposição à nicotina, o tempo de curso da dessensibilização será governado pelas taxas de transição entre a conformação inativa e as outras conformações, mas de forma geral, devido aos estados dessensibilizados terem maior afinidade pelos agonistas, após um tempo, todos os receptores podem ser dessensibilizados (Wang e Sun, 2005).

A *upregulation* de nAChRs, que pode ser definida como o aumento do número e/ou afinidade de nAChRs, é observada em cérebros de fumantes (Wüllner et al., 2008) e animais expostos à nicotina (Abreu-Villaça et al., 2003a). De fato, foi demonstrado que exposições por curtos períodos de tempo (2 dias) e com doses que produzem níveis plasmáticos de nicotina correspondentes a fumar 2 cigarros, já causam *upregulation* de nAChRs (Abreu-Villaça et al., 2003a). Acredita-se que a *upregulation* constitui uma resposta celular à dessensibilização (Fenster et al., 1999). Contudo, não se sabe se as mudanças ocorridas são acompanhadas por um aumento de função dos receptores (Buisson e Bertrand, 2002).

A *upregulation* dos nAChRs parece ser extremamente consistente entre espécies e idades de exposição. Este efeito já foi demonstrado em cultura de células (Ke et al., 1998), roedores (Abreu-villaça et al., 2003a; Doura et al., 2008; Marks et al., 1983), macacos (Slotkin et al., 2002) e, como já mencionado, em humanos (Perry et al., 1999; Wüllner et al., 2008). Em cérebros de roedores, a *upregulation* foi demonstrada pela primeira vez em 1983 (Marks et al., 1983). Estudos subseqüentes em humanos demonstraram *upregulation* em estruturas corticais e subcorticais, onde córtex cerebral, hipocampo e cerebelo apresentaram

as maiores alterações (Perry et al., 1999). Além disso, a *upregulation* tem sido evidenciada em diferentes idades de exposição à nicotina. Particularmente, tem sido demonstrado que a exposição durante o desenvolvimento promove efeitos mais intensos e duradouros. Interessantemente, tem sido constantemente demonstrado que a exposição à nicotina na adolescência promove a *upregulation* de modo mais robusto e persistente quando comparados com adultos (Abreu-Villaça et al., 2003a; Doura et al., 2008; Trauth et al., 1999).

Outro aspecto importante é que a *upregulation* ocorre em magnitudes diferentes dependendo do subtipo de receptor nicotínico. Particularmente, o subtipo α4β2 tem se mostrado o mais comum e sensível que sofre *upregulation* após exposição à nicotina (Abreu-Villaça et al., 2003a, 2004; Nashmi et al., 2003). Os receptores α4β2 são os subtipos de receptores nicotínicos mais abundantes presentes no cérebro de mamíferos (Buisson e Bertrand, 2002; Whiting e Lindstrom, 1987; 1988), sendo associado a esse subtipo muitas funções cognitivas, incluindo aprendizado/memória (Chan et al., 2007). Além disso, evidências originadas de estudos usando camundongos geneticamente modificados, nos quais as subunidades α4 e β2 foram deletadas, sugerem a participação dos α4β2 nAChRs no processo de estimulação da via mesolímpica dopaminérgica pela nicotina (Rollema et al., 2007). Conseqüentemente, esse subtipo de receptor tem se tornado um alvo molecular para a criação de novos agentes terapêuticos para a interrupção do hábito de fumar (Rollema et al., 2007).

1.2. Etanol

"ADMITIMOS QUE ÉRAMOS IMPOTENTES PERANTE O ÁLCOOL - QUE TÍNHAMOS PERDIDO O DOMÍNIO SOBRE NOSSAS VIDAS."

1º Passo (Livros "Os 12 passos e as 12 tradições" – Alcoólicos anônimos)

O etanol é um solvente orgânico que está entre as drogas não terapêuticas mais consumidas no mundo. Julgado em base molar, seu consumo é muitas vezes maior que o das demais drogas. O etanol em grandes doses pode, além de gerar vício, exercer efeitos prejudiciais em diversos órgãos e sistemas do organismo, como por exemplo, no fígado, sistema cardiovascular, rins e inclusive no sistema nervoso. Nos Estados Unidos da América, o consumo de etanol é a terceira causa de morte evitável (McGinnis e Foege, 1993). No Brasil, apesar da escassez de estudos, estima-se que 10% da população seja dependente do etanol (Laranjeira e Pinsky, 1997; Moreira et al., 1996). Além disso, o etanol é um potente agente teratogênico (Hannigan e Armant, 2000).

De forma interessante, o consumo de etanol normalmente se inicia durante o período da adolescência (Courtney e Polich, 2009). Hill e colaboradores (2000) demonstraram que também é na adolescência que os indivíduos se tornam consumidores regulares de etanol (consumo maior que uma vez por mês). Outros estudos epidemiológicos sugerem que a idade na qual se inicia o consumo de álcool pode influenciar profundamente na probabilidade de desenvolver transtornos no consumo de substâncias de abuso em momentos futuros (Clark et al., 1998; Duncan et al., 1997). O início precoce do uso de etanol tem sido mostrado em diversos estudos prospectivos e retrospectivos como um poderoso preditor de uso tardio e dependência de etanol (Deykin et al., 1987; Fergusson et al., 1994; Friedman e Humphrey, 1985; Hawkins et al., 1997; Prescott e Kendler, 1999; Rachal et al., 1982). Além disso, os adolescentes são mais sensíveis aos efeitos do etanol quando comparados aos adultos (Slawecki e Roth, 2004; Spear e Varlinskaya, 2005; Yttri et al., 2004). Existem evidências

que mostram que o uso de etanol durante a adolescência promove danos cognitivos mais severos e maior dano celular cerebral (Slotkin, 2002; Spear, 2000). Desta forma, tem sido proposto que diferentes taxas de desenvolvimento dos sistemas neurais envolvidos em diferentes efeitos do etanol podem contribuir para o mosaico de diferenças de idade em relação à sensibilidade ao etanol (Spear e Varlinskaya, 2005).

1.2.1. Metabolismo

O etanol, por ser altamente lipossolúvel, é rapidamente absorvido, sendo um percentual considerável no estômago. A maior parte do etanol é-metabolizada pelo fígado em acetaldeído através da enzima álcool desidrogenase, sendo apenas de 2-10% eliminado de modo inalterado pela urina, respiração e suor. Em média, na população geral, a álcool desidrogenase diminui a concentração do etanol no sangue em torno de 5 mmol/L de etanol por hora (equivalente a um drinque por hora) (Schuckit, 2006). O acetaldeído é um composto reativo e tóxico, que promove hepatotoxidade e também pode contribuir para os efeitos do consumo de etanol sobre o sistema nervoso (Deng e Deitrich, 2008; Quertemont et al., 2005; Hunt, 1996). Quase todo acetaldeído é convertido em acetato no fígado pela enzima aldeído desidrogenase. Os polimorfismos dessas enzimas tem sido estudados como possíveis preditores genéticos para a susceptibilidade do indivíduo apresentar vício pelo etanol (Dickson et al., 2006; Duranceaux et al., 2006).

1.2.2. Efeitos neuroquímicos

O etanol tem como sítios de ação uma grande variedade de alvos nas membranas celulares, como também, cascatas de sinalização intracelulares. Um dos efeitos mais importantes do etanol é sua propriedade de alterar funções dos receptores ionotrópicos (Larsson e Engel, 2004). Esta família de receptores inclui os receptores inibitórios da glicina e GABAA e os receptores excitatórios de NMDA do glutamato, serotonina (5-HT3), como também, os nAChRs (Larsson e Engel, 2004; Lovinger, 1999). Desta forma, o etanol promove alterações funcionais através de interações dose-dependentes em diversos sistemas neurais, incluindo GABAérgico, glutamatérgico, dopaminérgico, serotonérgico e colinérgico (Eckardt et al., 1998). Para GABAA e receptores de glicina, tem sido demonstrado que o etanol promove aumento de função; além disso, atua como co-agonista dos receptores 5-HT3 e pode bloquear as respostas mediadas pelo receptor de NMDA do glutamato (Lovinger, 1999; Lovinger et al., 1989; Mihic et al., 1997). Interessantemente, muitos desses sistemas neurais ainda estão em maturação durante a adolescência, o que poderia justificar o fato de o cérebro adolescente apresentar grande susceptibilidade aos efeitos do etanol (Carpenter-Hyland e Chandler, 2007; Schepis et al., 2008).

A função dos receptores de NMDA já é afetada em baixas doses de etanol, sendo sua resposta altamente dependente da concentração estudada e do subtipo de receptor envolvido. Um estudo de *patch-clamp*, realizado por Lima-Landman e Albuquerque (1989), demonstrou que concentrações muito baixas de etanol (1,74–8,65 mM) aumentam a probabilidade da abertura do canal iônico após ativação pelo NMDA em cultura de células hipocampais, mas sem afetar o tempo de abertura do canal, enquanto que em altas concentrações (86.5–174 mM) promove diminuição da probabilidade de abertura do canal, como também, reduz o tempo de abertura. Todas as subunidades dos receptores de NMDA têm se mostrado sensíveis

aos efeitos do etanol, porém, os canais N1-NR2C e NR1/NR2D apresentam menos afinidade que as combinações NR1-NR2A e NR1-NR2B (Kuner et al., 1993; Masood et al., 1994; Yang et al., 1996). Desta forma, a distribuição tecidual destes subtipos receptores pode contribuir para explicar as consideráveis variações do efeito do etanol sobre diferentes regiões cerebrais. A inibição da via excitatória glutamatérgica pelo etanol, contribui possivelmente para seus efeitos ansiolíticos, como também, pode ser uma importante causa dos efeitos neurotóxicos (Tsai e Coyle, 1998), particularmente intoxicação, sintomas de abstinência (Koob et al., 1998) e episódios de perda de memória (Tsai e Coyle, 1998).

Os receptores GABAérgicos estão entre os mais importantes sítios de ligação para os efeitos comportamentais do etanol, principalmente em relação aos seus efeitos ansiolíticos. Entretanto, os dados da literatura a respeito da modulação do etanol nestes receptores têm sido controversos (Moriguchi et al., 2007). Dados na literatura sugerem que a potencialização da atividade do receptor GABA depende da combinação das subunidades dos receptores (Wafford e Whiting, 1992; Wafford et al., 1990, 1991), viabilidade celular (Aguayo et al., 2002), tipos celulares (Mori et al., 2000; Sapp e Yeh, 1998) e processamentos que ocorrem após a tradução, incluindo fosforilação do receptor (Harris et al., 1995). A sensibilidade do sistema GABAérgico de certas áreas do cérebro para os efeitos do etanol possivelmente são relevantes na geração dos seus efeitos ansiolíticos e amnésicos, como também, em altas doses, deve possuir um grande papel na produção de sedação (Little, 1999).

Em relação aos receptores de serotonina, os efeitos do etanol também parecem depender do subtipo de receptor estudado. O etanol em altas doses potencializa a função dos receptores de serotonina 5-HT3 em células de neuroblastoma (Lovinger, 1991). Outros estudos têm demonstrado que outros subtipos de receptores para serotonina podem ser inibidos pelo etanol, como, por exemplo, foi demonstrado que o etanol pode promover a inibição da função do subtipo 5HT1C em oócitos (Frye et al., 1991). Antagonistas dos

receptores 5-HT3 têm sido usados terapeuticamente como agentes antieméticos, por dedução, tem sido sugerido que a ação do etanol em potencializar as respostas do 5-HT3 poderia contribuir para a náusea e vômitos provocados pela alta ingestão de álcool (Little, 1999). Além disso, tem sido sugerido que o sistema serotoninérgico está envolvido na resposta de recompensa pelo uso do etanol (Sellers et al., 1992).

De particular interesse para o presente estudo, os nAChRs têm sido reconhecidos como importantes sítios de ação do etanol. Estudos comportamentais em roedores têm demonstrado grande interação entre o etanol e os receptores nicotínicos. Além disso, Wu e colaboradores (1993) sugerem que o etanol estabiliza o estado aberto do nAChR, como também tem sido demonstrado que o etanol pode aumentar a finidade dos nAChRs pelos seus agonistas. Como acontece para outros receptores, o efeito do etanol pode variar de acordo com a sensibilidade dos subtipos de receptores. Em estudos usando diferentes subtipos de nAChRs expressados em Xenopus oocytes, o etanol tende aumentar a resposta da nicotina nos subtipos $\alpha_2\beta_2$, $\alpha_3\beta_2$, e $\alpha_4\beta_2$ (de Fiebre et al., 1995). Quando avaliados os receptores α_7, resultados contraditórios são encontrados. Tanto inibição (Yu et al., 1996) como potencialização (Covernton e Connolly, 1997) tem sido encontrados, ilustrando as complexas interações entre o etanol e os nAChRs. Estes resultados sugerem que o nAChR pode significar um denominador comum para as interações entre nicotina e etanol, explicando o grande co-consumo destas drogas nos seres humanos. A ativação de nAChRs têm mostrado aumentar a liberação sináptica de GABA, glutamato, e outros neurotransmissores (Alkodon et al.,1997, 1999, 2000; Genzen e McGehee et al., 2003, 2005; Lindstrom, 1997; Mansvelder et al., 2002; Yang et al., 1996). Desta forma, a modulação dos nAChRs causada pelo etanol pode levar a uma cascata de eventos sinápticos envolvendo múltiplos neurotransmissores, que resulta em várias mudanças comportamentais.

Diversos efeitos comportamentais do etanol parecem requerer estimulação dos sistemas dopaminérgicos. Neste sentido, amplas evidências indicam que o sistema mesocorticolímbico dopamérgico está envolvido no sistema de recompensa cerebral e que a dopamina está direta ou indiretamente envolvida na ação de reforço aguda do etanol em animais experimentais assim como em humanos (Engel et al., 1999; Koob, 1992; Tupala et al., 2001; Volkow, 1996; Wise e Rompre, 1989).

1.3. Co-uso e Co-abuso

O etanol e a nicotina são as drogas mais comumente usadas no mundo (Dani e Harris, 2005). Além disso, tem sido amplamente relatado em estudos epidemiológicos que existe uma consistente relação entre o tabagismo e consumo de etanol (Larsson e Engel, 2004; Miller e Gold, 1998). Diversos estudos têm demonstrado que 80% a 90% dos alcoolistas fumam (Burling e Ziff, 1988; Crowley et al., 1974). O consumo de etanol entre fumantes é duas vezes maior do que entre os não fumantes (Carmody et al., 1985) e estima-se que o alcoolismo seja de 10 a 14 vezes mais comum entre fumantes quando comparados com não fumantes (Di Franza e Guerrera, 1990), além do consumo diário de cigarros por alcoolistas ser maior do que o consumo por fumantes não alcoolistas (Rezvani e Levin, 2002). Como mencionado anteriormente, o uso de ambas as drogas normalmente se inicia durante o período de adolescência (Nelson et al., 1995; Spear, 2000). Além disso, estudos em adolescentes têm demonstrado uma forte correlação entre a idade de início do tabagismo e a susceptibilidade ao vício pelo etanol (Grant, 1998), caracterizando a adolescência como período de vulnerabilidade.

O fato do consumo de etanol e o tabagismo serem legalmente permitidos pode contribuir para o co-uso e co-abuso. Entretanto, tem crescido o número de trabalhos que indicam que fatores biológicos podem contribuir para explicar a freqüente associação do uso entre as duas drogas. Conseqüentemente, a forte associação entre o consumo de etanol e o fumo de cigarros está provavelmente atribuída a múltiplos fatores, incluindo ações farmacológicas comuns a ambos (Little, 2000). Muitos mecanismos tem sido sugeridos. Uma droga pode promover um aumento da motivação para o consumo de outra droga por atuar em um mesmo alvo cerebral responsável pelo efeito de reforço positivo de ambas as drogas. A geração de tolerância cruzada poderia reduzir os efeitos aversivos da droga e motivar as pessoas a usarem outras drogas que gerem o mesmo efeito de recompensa. Além disso, estudos recentes sugerem que componentes genéticos podem predispor o uso combinado do etanol e da nicotina (Funk et al., 2006).

1.3.1. Metabolismo

A possibilidade de interação farmacocinética entre nicotina e etanol poderia ajudar a explicar a forte associação do uso entre ambas. Por exemplo, se a presença de nicotina aumentar o metabolismo do etanol, desta forma diminuindo sua concentração circulante, poderia ocorrer diminuição dos efeitos de recompensa promovidos pelo etanol. Neste sentido, é fundamental conhecer o quanto uma droga pode ter o seu metabolismo afetado pela presença da outra.

A literatura apresenta dados controversos em relação à existência de interação farmacocinética para nicotina e etanol em animais, sendo provável que essa inconsistência seja devida à variação da forma de administração da droga entre os estudos. De fato, foi

demonstrado que os níveis plasmáticos de etanol, quando este é administrado via oral, são reduzidos em animais expostos concomitantemente a nicotina (Chen e Harle, 2005; Gilbertson e Barron, 2005). Entretanto quando a administração do etanol é realizada por via intraperitoneal (i.p.), a concentração plasmática de etanol não foi afetada pela exposição à nicotina (Parnell et al., 2006). Collins e colaboradores (1988) também demonstraram que a exposição à nicotina não afeta a taxa de eliminação de etanol.

O mecanismo que explica a habilidade da nicotina em reduzir a concentração sanguínea de etanol quando este é administrado via oral, ainda não está completamente compreendido. Existem evidências na literatura que sugerem que a nicotina promove redução do esvaziamento gástrico (Nowak et al., 1987; Scott et al., 1993), possivelmente por induzir relaxamento da musculatura gástrica (Tugay et al., 2003). Desta forma, o álcool fica retido no estômago por um tempo maior, sendo sujeito à ação da enzima álcool desidrogenase presente no estômago. Como a maioria da absorção do etanol é realizada pelo intestino delgado (Johnson et al., 1991), haveria uma redução da absorção do etanol e, conseqüentemente, uma menor concentração plasmática.

1.3.2. Neuroquímica do co-uso e co-abuso

Devido a grande complexidade que pode envolver os estudos de interação entre nicotina e etanol, onde fatores genéticos e psicossociais podem contribuir para explicar o co-uso e co-abuso, a existência de modelos animais é fundamental para estudar a importância das interações fisiológicas. De forma interessante, a maioria dos estudos animais estão de acordo com as evidências encontradas em humanos. Diversos estudos têm demonstrado que a exposição à nicotina promove aumento da auto-administração de etanol em animais (Larsson e Engel, 2004; Lé et al. 2000; Smith et al. 1999). Em modelos animais de intoxicação pelo

etanol, foi demonstrado que nicotina atenua o aumento na utilização da glicose no cerebelo de camundongos (Anwer e Dar, 1995), a descoordenação motora (Dar et al., 1994), assim como, reduz o efeito do etanol sobre a memória (Tracy et al., 1999). Além disso, tem sido demonstrada tolerância cruzada entre as drogas. De Fiebre e Collins (1993) demonstraram que a administração crônica do etanol, a qual induz tolerância para vários efeitos do etanol, também confere tolerância para os efeitos de hipotermia e bradicardia gerados pela nicotina em camundongos e vice-versa.

A maioria dos trabalhos sobre mecanismos neurais que tentam explicar a interação entre drogas de abuso, incluindo a nicotina e o etanol, foca seus estudos no funcionamento do sistema mesolímbico dopaminérgico. Este sistema é uma via crucial para a ação de drogas de abuso, estando associado com as sensações de recompensa. Embora os mecanismos exatos da ação de drogas de abuso no sistema mesolímbico dopaminérgico não estejam estabelecidos, acredita-se que estas drogas, atuando direta ou indiretamente em células dopaminérgicas, causem alterações persistentes, as quais seriam responsáveis, pelo menos em parte, pelas reações adversas associadas à abstinência. De forma geral, estudos demonstram que tanto a nicotina quanto o etanol promovem aumento da liberação de dopamina no núcleo acumbente (Carr et al., 1989; Blomqvist et al., 1992). Blomqvist e colaboradores (1992) mostraram que o efeito do etanol em aumentar a atividade motora, como também a liberação de dopamina no núcleo acumbente, pode ser prevenido pelo uso da mecamilamina, um antagonista nicotínico. Desta forma, o efeito da nicotina e também do etanol sobre o sistema mesolímbico dopaminérgico depende da ativação dos nAChR. Considerando os resultados mencionados acima, assim como, a capacidade do etanol interagir com os nAChRs, é possível especular que o sistema colinérgico central seja o sítio onde a nicotina e o etanol interagem, podendo explicar, pelo menos em parte, a existência do co-uso e co-abuso.

Estudos epidemiológicos também sugerem que as interações entre nicotina e o etanol podem acontecer durante o período de retirada das drogas. Por exemplo, a interrupção do ato de fumar é mais difícil de realizar por indivíduos que abusavam ou abusam do etanol (Bobo et al., 1987). Além disso, a interrupção do tabagismo tem sido associada com a alteração do consumo de etanol, podendo aumentar esse consumo (Carmelli et al., 1993) ou diminuí-lo (Friend e Pagano, 2005). Alguns trabalhos têm sugerido que o uso de nicotina (no tabaco) pode diminuir os sintomas da abstinência pelo etanol (Lallemand et al., 2007).

A existência de modelos animais abre grandes possibilidades de investigar as interações da nicotina e do etanol na abstinência, porém são também raros os estudos de interação durante o período de retirada. Ratos em abstinência de etanol são mais sensíveis ao efeito da nicotina na geração de tremores (Gothoni 1983; Gothoni e Ikola 1985). Lallemand e colaboradores (2006) identificaram que a administração de nicotina promoveu diminuição significativa da liberação de glutamato no núcleo acumbente nas primeiras 24 horas de retirada da exposição crônica ao etanol. Interessantemente, tem sido proposto que β2*-nAChRs contribuem para a geração dos distúrbios afetivos gerados pela retirada da nicotina (Jackson et al., 2008), como também, os α4*-nAChRs parecem modular os efeitos da abstinência pelo etanol (Butt et al., 2004). Estes resultados sugerem que o sistema colinérgico também pode ser um sítio de interação entre nicotina e etanol durante o período de retirada. Em acordo, resultados prévios do nosso laboratório demonstraram em camundongos existir interação entre essas drogas na ansiedade e no aprendizado/memória, comportamentos estes associados com a função do sistema colinérgico (Abreu-Villaça, 2007; 2008).

1.4. A adolescência como período de vulnerabilidade

A adolescência inclui todo processo de transição da infância para a idade adulta. O limite temporal da adolescência não é de fácil definição. Em algumas espécies é difícil caracterizar quando a transição para adolescência começa a acontecer e quando o indivíduo passa de adolescente a adulto. Em roedores como ratos e camundongos, as primeiras alterações associadas à adolescência podem ocorrer já a partir do vigésimo dia de vida pós-natal (PN20) e as últimas mudanças podem ocorrer até PN55. Em primatas não humanos, a adolescência ocorre geralmente entre 2 e 4 anos de vida (Lewis, 1997); em humanos, a adolescência é comumente definida como a segunda década de vida (Petersen et al., 1996).

Este período é caracterizado por dramáticas mudanças corporais e comportamentais. Estudos com roedores têm mostrado que adolescentes exibem diferenças quando comparados com adultos em relação a medidas de ansiedade, depressão e reatividade ao estresse (Adriani e Laviola, 2004; Slawecki, 2005). Durante adolescência, roedores apresentam elevados níveis de busca pela novidade (Adriani et al., 1998), impulsividade (Adriani e Laviola, 2003) e comportamento de risco (Macri'et al., 2002), assim como redução de resposta ao estresse (Adriani e Laviola, 2000). Em humanos, a adolescência é caracterizada por traços comportamentais como labilidade emocional, impulsividade e comportamento de risco que estão associadas com o aumento de vulnerabilidade para transtornos neuropsiquiátricos, incluindo doenças afetivas e susceptibilidade ao vício (Andersen, 2003; Arnett, 1999; Spear, 2000; Teicher et al., 2003; Volkow e Li, 2005; Wallace et al., 2003).

O cérebro apresenta maior grau de desenvolvimento durante a infância e relativa estabilidade na idade adulta, sendo seu desenvolvimento pontuado por rápidas transformações neurais durante a adolescência (Spear, 2000). De fato, fenômenos como neuroproliferação, apoptose e rearranjo sináptico continuam durante a adolescência. (Bayer, 1983; Bayer et al,

1982). De particular interesse para o presente estudo, a maturação do sistema colinérgico central, envolvido com o aprendizado/memória e repostas a psicoestimulantes, é consolidada durante o período da periadolescência (Matthews et al, 1974; Nadler et al, 1974; Zahalka et al, 1993). Regiões neurais como o córtex pré-frontal e outras regiões de projeções dopaminérgicas do prosenencéfalo, apresentam alterações proeminentes durante a adolescência. Dada a importância dessas regiões cerebrais na modulação de mecanismos de recompensa associados ao uso de drogas (Koob, 1992) e de respostas a agentes estressores (Dunn e Kramarcy, 1984) e a associação entre ambos (Goeders, 1997; Piazza et al., 1991), não é surpresa constatar que adolescentes respondem de formas diferentes a drogas de abuso, agentes estressores e a interação entre ambos, quando comparados com animais adultos ou mais maduros (Spear, 2002). De fato, adolescentes apresentam grande probabilidade de experimentar drogas de abuso, incluindo tabaco e etanol (Grant et al., 1987; Nelson et al., 1995; Webster et al., 1994), e desenvolver vício que adultos (Maggs et al., 2008). Além disso, parece existir uma forte correlação entre a idade inicial do uso da droga e a probabilidade de apresentar vício quando adulto (DiFranza e Guerrera, 1990), como também, o uso de uma droga na adolescência parece abrir "as portas" para o uso de múltiplas drogas de abuso no decorrer da vida (Kandel et al., 1992). Para várias drogas de abuso, incluindo a nicotina e o etanol, indivíduos que não iniciaram o uso durante a adolescência raramente iniciam o uso em idades posteriores (Chen e Kandel, 1995; Kandel e Logan, 1984).

Além da imaturidade das estruturas cerebrais envolvidas com o estabelecimento de vício, vários outros fatores podem ajudar a explicar o motivo da alta associação do uso de drogas de abuso e a adolescência. Neste sentido, destacam-se: o padrão de comportamento, a menor intensidade de efeito negativo da droga e maiores efeitos de recompensa. Dois comportamentos envolvidos na iniciação do uso de drogas são a impulsividade ou um desejo por novidades e experiências excitantes (*sensation seeking*). Intensa busca pela novidade,

característica do período da adolescência, tem sido associada a um maior consumo de drogas de abuso (Abreu-Villaça et al., 2006; Spear, 2000). Estudos em humanos têm encontrado forte correlação entre altas taxas de busca por sensações novas e uso de álcool e nicotina (Zuckerman, 1994). Adicionalmente, a dependência ao álcool e à nicotina tem sido associada a altos níveis de impulsividade (Mitchell, 1999; Poulos et al., 1998). Em ratos adolescentes, nicotina e etanol produzem efeitos agudos menos pronunciados, como também, apresentam uma resposta mais branda à retirada (Levin et al., 2003; Spear, 2002). Como exemplo, ratos adolescentes, sob a influencia do etanol, são menos sensíveis a desenvolver as desordens motoras ou a ficarem sedados (Silveri e Spear, 1998; White et al., 2002). Da mesma forma, ratos adolescentes possuem menor sensibilidade aos efeitos da nicotina sobre a atividade motora quando comparados com adultos (Vastola et al., 2002).

Ao contrário do fato de apresentar mais recompensa e menos efeitos adversos, adolescentes são mais susceptíveis aos efeitos deletérios do uso das drogas de abuso, principalmente ao álcool. Evidências demonstram que consumo de etanol durante a adolescência causa maiores déficits cognitivos quando comparados com a vida adulta (Spear, 2000). White e Swartzwelder (2004) demonstraram que o hipocampo de ratos adolescentes é altamente susceptível a inibição dos potenciais de longa duração pelo etanol, tornando os ratos adolescentes altamente sensíveis aos danos na memória gerados pelo etanol.

Tomadas em conjunto, estas evidências sugerem que a adolescência é um período de vulnerabilidade ao consumo de etanol e ao tabagismo, e desta forma, reflete na possibilidade da existência de vias neuroquímicas, particularmente sensíveis durante a adolescência, compartilhadas entre as duas drogas. O desenvolvimento de modelos animais de exposição a drogas de abuso durante a adolescência tem sido extremamente interessante, pois permite a investigação destas questões (Barron et al., 2005).

1.5. Estudos animais sobre a interação nicotina e etanol durante a adolescência

A grande maioria da literatura que aborda o período de adolescência tem estudado a susceptibilidade desta idade em relação aos adultos para cada droga separadamente, sendo extremamente escassos os estudos onde é considerada a interação entre drogas. Particularmente, o nosso laboratório tem contribuído para ocupar essa lacuna na literatura. Usando um modelo da dupla exposição de etanol e nicotina na adolescência de camundongos, o nosso grupo de pesquisa estudou os efeitos sobre a ansiedade (Abreu-Villaça et al., 2007), aprendizado/memória (Abreu-Villaça et al., 2008) e neurotoxicidade (Oliveira-da-Silva et al., 2009 *in press*).

Usando o teste do labirinto em cruz elevado, modelo amplamente utilizado na avaliação dos níveis de ansiedade, foi demonstrado que o etanol promove diminuição da ansiedade na adolescência de camundongos e que a nicotina pode reverter este efeito. Além disso, esse estudo indicou que o etanol e a nicotina podem interagir após longo tempo de retirada na idade adulta, promovendo aumento da ansiedade (Abreu-Villaça et al., 2008). Também observamos interação entre as drogas na regulação da resposta comportamental quando considerado aprendizado/memória. Usando o modelo de esquiva passiva, foi demonstrado que a nicotina e o etanol quando expostos em conjunto promovem danos na memória significativamente maiores que cada droga individualmente em camundongos adolescentes (Abreu-Villaça et al., 2007). Neste mesmo estudo, também se verificou que após pequeno tempo de retirada, a nicotina promoveu um aumento da capacidade de memória/aprendizado, mas que este efeito foi bloqueado pelo uso concomitante do etanol. Em relação a neurotoxocidade, recentemente foi observado que o etanol promove aumento da morte celular em todas as áreas do hipocampo de camundongos adolescentes, sendo ambas as populações de neurônios e glia afetados, enquanto que a nicotina causou aumento seletivo da

morte celular, sendo as camadas molecular e CA1 afetadas. Neste estudo também houve interação de efeitos quando as duas drogas foram expostas simultaneamente, só que neste caso, a exposição combinada promoveu efeitos menos severos na morte celular (Oliveira-da-Silva et al., 2009 *in press*).

Apesar dos estudos epidemiológicos e dos estudos em modelos animais dos efeitos combinados da nicotina e etanol sobre o comportamento e a neurotixicidade, pouco se sabe sobre a base neuroquímica das alterações promovidas pela co-exposição durante o período da adolescência. Considerando que a nicotina é um agonista dos nAChRs e que tem sido demonstrado que o etanol interage com receptores ionotrópicos, incluindo os colinérgicos, o estudo do sistema colinérgico possui grande relevância no entendimento das bases neuroquímicas envolvidas nos processos de co-abuso e dependência dessas drogas.

2. OBJETIVOS

Estudar os efeitos da exposição à nicotina e/ou ao etanol no sistema colinérgico durante a adolescência, durante a retirada e, para avaliar os efeitos persistentes da exposição, também durante a idade adulta.

Para essa avaliação foram utilizados três biomarcadores colinérgicos:

- Ligação da [^{3}H]citisina ao $\alpha4\beta2$ nAChR. Subtipo de receptor nicotínico predominante no cérebro de mamíferos;
- Atividade da colina acetiltransferase (ChAT), responsável pela biosíntese de acetilcolina. ChAT é um marcador constitutivo para os terminais colinérgicos, sendo utilizado para avaliar a densidade de inervação colinérgica;
- Ligação do [^{3}H]hemicholinium-3 (HC-3) ao transportador pré-sináptico de colina, que reflete a atividade sináptica colinérgica.

Considerando que têm sido demonstradas diferenças entre machos e fêmeas para os efeitos cerebrais da nicotina e etanol (Abreu-villaça et al., 2007, 2008). Neste trabalho, também consideramos o sexo como fator.

Além disso, considerando que o nível de exposição é um fator importante na interpretação dos resultados e na possibilidade de se realizar generalizações à população humana, como também podem ocorrer importantes interações farmacocinéticas entre a nicotina e o etanol, foi avaliada a concentração plasmática de etanol e de cotinina, resíduo metabólico da nicotina.

3. RESUMO DA METODOLOGIA (DESENHO EXPERIMENTAL)

Para o total dos dois trabalhos apresentados nesta tese, foram utilizados 183 camundongos da cepa C57BL/6 (100 machos e 83 fêmeas) de 42 ninhadas. Os experimentos descritos a seguir foram previamente aprovados pelo Comitê de Ética para o Cuidado e Uso de Animais Experimentais do Instituto de Biologia Roberto Alcântara Gomes da UERJ e estão de acordo com a declaração de Helsinki e com o Guia de cuidados e uso de animais de laboratório adotado e promulgado pelo Instituto Nacional de Saúde.

O desenho experimental apresentado neste trabalho têm sido largamente utilizado pelo nosso grupo de pesquisa no estudo da exposição combinada à nicotina e ao etanol no comportamento e na função cerebral de camundongos adolescentes (Abreu-Villaça, 2007; 2008; Oliveira-da-Silva et al., 2009 *in press*). Desta forma, do 30º ao 45º dia de vida pós-natal (PN30-PN45), (-)-nicotina *free* base (50ug/ml) dissolvida em sacarina a 2% ou somente sacarina a 2% são administradas na garrafa de água enquanto que etanol (2g/kg) ou salina são injetados (i.p) em dias alternados. Desta forma, o estudo completo do impacto da exposição à nicotina e ao etanol durante a adolescência consta de 4 grupos experimentais: VEH (sacarina via oral + salina i.p), NIC (nicotina/sacarina via oral + salina i.p), ETOH (sacarina via oral + álcool i.p) e os animais que recebem a dupla exposição NIC+ETOH (nicotina/sacarina via oral + álcool i.p). Assim, com este desenho, somos capazes de detectar os efeitos da nicotina, do etanol, e da dupla exposição.

O sistema colinérgico foi estudado no córtex cerebral e mesencéfalo, sendo estas regiões escolhidas por estarem envolvidas com os processos de vício, recaída, e também com funções cognitivas. Tais áreas foram avaliadas durante o período de exposição (PN45), no período de retirada (PN50) e durante a idade adulta (PN75). Nestes períodos de estudo, os animais sofreram eutanásia, seus cérebros foram dissecados e congelados rapidamente por

imersão em nitrogênio líquido e, então, guardados a -45ºC para posterior análise bioquímica. O sangue foi coletado em PN45, sendo o plasma congelado a -20ºC para posterior quantificação da cotinina. Soro foi coletado em PN44 e mantido a 4ºC para a quantificação dos níveis de etanol. Em resumo, a figura 2 apresenta a linha temporal dos principais procedimentos realizados neste desenho experimental.

Maiores detalhes metodologia e os resultados serão apresentados na seqüência através dos artigos que foram gerados a partir desta Tese.

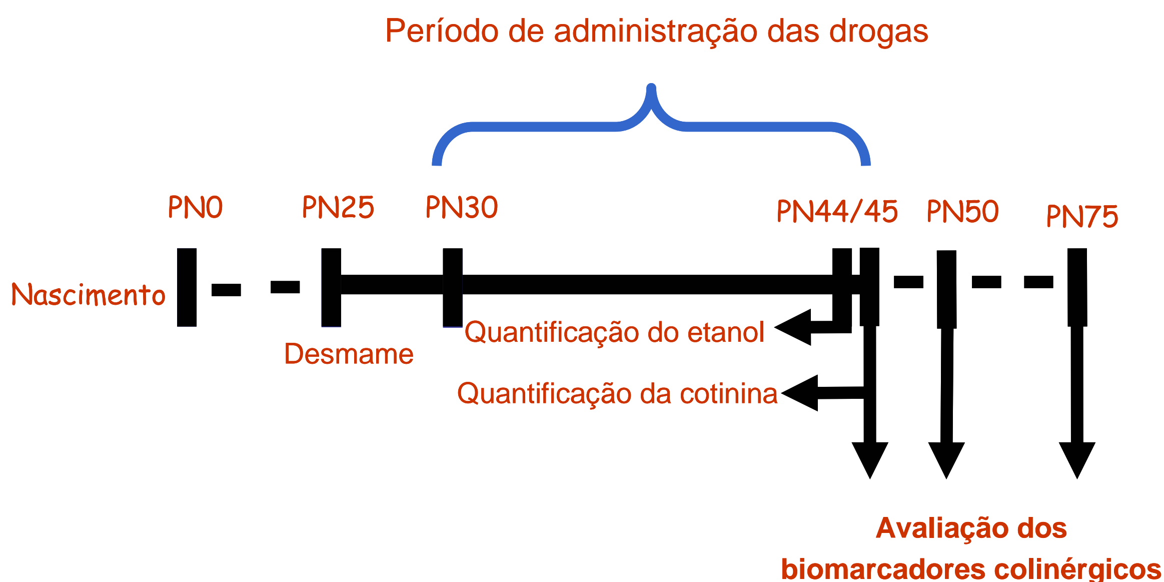

Figura 1 – Linha temporal do modelo experimental utilizado.

4. ARTIGO 1 – Efeitos da exposição combinada de nicotina e etanol no sistema colinérgico de camundongos adolescentes.

Este primeiro trabalho foi publicado em 2008 no periódico internacional *Brain Research* (fator de impacto 2008=2,494), sendo intitulado: "Nicotine and ethanol interact during adolescence: effects on the central cholinergic systems" (2008, 1232:48-60). Além disso, os resultados deste trabalho foram apresentados em 2 congressos internacionais: no *IBRO/LARC* (I Congresso de Neurociências da América Latina, Caribe e Península Ibérica) e no *Society for Neuroscience Internacional Meeting* (38th annual meeting of the Society for Neuroscience). Foi trabalho premiado (2º lugar) no I Congresso de Biólogos dos Estados do Rio de Janeiro e Espírito Santo (I CBio).

Em resumo, foi avaliado o efeito da nicotina e/ou etanol no sistema colinérgico ao final do período de exposição durante adolescência. Neste artigo demonstramos que, em ambas as regiões estudadas (córtex cerebral e mesencéfalo), a exposição combinada NIC+ETOH promoveu um efeito mais robusto na geração de *upregulation* dos nAChRs quando comparado com a nicotina ou o etanol separadamente. Em relação à atividade da ChAT, a exposição combinada foi capaz de reverter os efeitos promovidos por cada uma das drogas. Estes resultados indicam que o sistema colinérgico central é um sítio de interação entre a nicotina e o etanol durante a adolescência.

BRAIN RESEARCH 1232 (2008) 48–60

available at www.sciencedirect.com

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Nicotine and ethanol interact during adolescence: Effects on the central cholinergic systems

*Anderson Ribeiro-Carvalho, Carla S. Lima, Cláudio C. Filgueiras, Alex C. Manhães, Yael Abreu-Villaça**

Laboratório de Neurofisiologia, Departamento de Ciências Fisiológicas, Instituto de Biologia Roberto Alcântara Gomes, Centro Biomédico, Universidade do Estado do Rio de Janeiro, Av. Prof. Manoel de Abreu 444, 5 andar, Vila Isabel, Rio de Janeiro, RJ, 20550-170, Brazil

ARTICLE INFO

Article history:
Accepted 17 July 2008
Available online 28 July 2008

Keywords:
Adolescence
Nicotinic cholinergic receptor
Alcohol
Tobacco
Development

ABSTRACT

Co-occurrence of tobacco smoking and alcohol consumption during adolescence is frequent and well documented. However, little is known about the basic neurobiology of the combined exposure in the adolescent brain. Since nicotine is a cholinergic agonist and it has been shown that ethanol interferes with nicotinic acetylcholine receptors (nAChRs), the current work focused on cholinergic systems. From the 30th to the 45th postnatal day (PN), C57BL/6 male and female mice were exposed to nicotine free base (NIC) and/or ethanol (ETOH). Four groups were analyzed: 1) concomitant NIC (50 μg/ml in 2% saccharin to drink) and ETOH (25%, 2 g/kg i.p. injected every other day) exposure; 2) NIC exposure; 3) ETOH exposure; 4) vehicle. We assessed nAChR binding, choline acetyltransferase (ChAT) activity and [^{3}H]hemicholinium-3 (HC-3) binding in the cerebral cortex and midbrain of mice on PN45. In the cortex, ETOH had no effect on nAChRs. In contrast, NIC produced nAChR upregulation while NIC+ETOH elicited a more pronounced effect. In the midbrain, neither ETOH nor NIC had effects on nAChRs. NIC+ETOH, however, elicited a robust nAChR upregulation. Regarding ChAT activity, treatment effects differed between males and females in the cortex. Male NIC mice presented an increase in ChAT. However, ETOH reversed this effect. In contrast, female NIC mice presented decreased ChAT activity. In the midbrain, ETOH increased ChAT. HC-3 binding was not affected. These results indicate that the central cholinergic system is a site at which nicotine and ethanol interact. This interaction might underlie the association between tobacco and alcohol consumption during adolescence.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Tobacco and ethanol are among the most widely used drugs in the world. Furthermore, co-occurrence of tobacco smoking and alcohol consumption is frequent and well documented. Among alcoholics, smoker rates of more than 80% have been shown while 30% of smokers are alcoholics (Batel et al., 1995; Miller and Gold, 1998). Individuals that are alcohol-dependent present a higher frequency of nicotine-dependence than those that do not drink (Hashimoto et al., 2001). Nicotine-dependence also seems to be more severe in the alcohol-dependent individuals (Marks et al., 1997). Smokers consume twice as much alcohol as do non-

* Corresponding author. Fax: +55 21 2587 6129.
E-mail address: yael_a_v@yahoo.com.br (Y. Abreu-Villaça).

Abbreviations: ANOVA, analysis of variance; ChAT, choline acetyltransferase; FPLSD, Fisher's Protected Least Significant Difference; HC-3, hemicholinium-3; nAChR, nicotinic acetylcholine receptor; PN, postnatal day; rANOVA, repeated-measures analysis of variance

0006-8993/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2008.07.062

smokers, and alcoholics who smoke consume more cigarettes than do non-alcoholic smokers (Carmody et al., 1985; Dawson, 2000; DiFranza and Guerrera, 1990; Larsson and Engel, 2004; Meyerhoff et al., 2006; Patten et al., 1996). Interestingly, it is now clear that adolescents show a peculiar sensitivity to nicotine and there are also evidences that alcohol use during adolescence leads to more severe cognitive deficits and brain cell damage (Slotkin, 2002; Spear, 2000a,b). Most cigarette smokers begin their habit as adolescents (National Institute on Drug Abuse, 1998; Nelson et al., 1995); epidemiological data have shown a great number of high school students smoking cigarettes—approximately one-third in the US (Centers for Disease Control and Prevention, 2000). Exploratory alcohol use also typically occurs during adolescence (Spear, 2000a,b) and, in 2000, more than 50% of high school students reported drinking alcohol (Johnston et al., 2001). Perhaps most importantly, studies of adolescent psychoactive substance use have confirmed that drinkers are more likely than nondrinkers to be smokers and vice versa (Chen and Kandel, 1995; Torabi et al., 1993; Schorling et al., 1994) and have also shown that there is a strong correlation between onset of tobacco consumption at an early age and alcohol addiction, characterizing adolescence as a period of vulnerability (Grant, 1998). Altogether, these findings indicate that a better understanding of the frequent association between tobacco smoking and alcohol consumption during adolescence is an important area of research since it could help elaborate intervention strategies to decrease the co-abuse.

Several mechanisms have been proposed as underlying this epidemiological association. These include genetic predisposition and/or environmental influence (Howard et al., 2003; Lê et al., 2006; Swan et al., 1990), cross-tolerance between ethanol and nicotine (Collins, et al., 1988, 1996) and putative pharmacokinetic interactions (Parnell, et al., 2006). Another strong contribution may result from pharmacodynamic interactions between drugs. In this regard, among tobacco components, nicotine has been shown to be responsible for a wide range of nervous system effects resulting from tobacco use, including tobacco addiction (Benowitz, 1992; Dani and Heinemann, 1996). As an acetylcholine analog, the ionotropic nicotinic acetylcholine receptors (nAChRs) are the primary cellular mediators of nicotine's effects. As for ethanol, it directly influences the function of various ligand-gated ion channels, including nAChRs (for review: Grant, 1994), which suggests that the nAChR is a site at which nicotine and ethanol interact. Corroborating this suggestion, it was demonstrated that ethanol enhances agonist-induced ion flux through nAChRs (Aistrup et al., 1999; Cardoso et al., 1999) and that ethanol-induced stimulation of locomotor activity and mesolimbic dopamine systems involve nAChR activation (Blomqvist et al., 1992; Soderpalm et al., 2000). Concomitant nicotine and ethanol exposure in adult rats results in additive dopamine release in the nucleus accumbens, an effect that seems to be mediated by nAChR (Tizabi et al., 2007). Additionally, in two previous studies, we found that nicotine and ethanol interact affecting anxiety levels and memory/learning during adolescence (Abreu-Villaça et al., 2007, 2008), behaviors that are known to be affected by cholinergic system dysregulation (Dani and Bertrand, 2007; Degroot and Treit, 2002; Engin and Treit, 2007; File, et al., 2000; Hasselmo, 2006; Ricceri et al., 2002; Viveros et al., 2007).

Despite these findings, there have been relatively few animal studies on the basic neurobiology of the combined nicotine and ethanol exposure. Most importantly, there have been scant studies in animal models of adolescent exposure, the period at which both smoking and drinking most frequently begin. Since nicotine is a cholinergic agonist and since it has been shown that alcohol interferes with nAChRs, the purpose of the current study was to examine the effects of adolescent nicotine and/or ethanol administration on the central cholinergic neurotransmitter system. Regular smokers tend to smoke intermittently in order to maintain blood levels of nicotine above a minimum effective concentration during their active period. Therefore, in keeping with earlier experimental designs (Abreu-Villaça et al., 2006, 2007, 2008), we chose to give animals free access to a nicotine solution in the drinking water, which allows for consumption during their active time. For ethanol, we chose a moderate dose to be injected (i.p.) every other day, mimicking adolescents' binge drinking. Ethanol i.p. injections during adolescence were shown to be effective in eliciting behavioral (Lopez et al., 2003; Song et al., 2008) and neurochemical alterations (Sircar and Sircar, 2006; Jang et al., 2002b) in rodents. In light of the unique behavioral sensitization due to nicotine and ethanol exposure during adolescence (Abreu-Villaça et al., 2007, 2008), exposure began on postnatal day (PN) 30, which, based on brain development, onset of puberty, and patterns of drug reactivity, represents the early adolescent stage in the rodent, and extended up to PN45, still well within adolescence (Spear, 2000a,b). We evaluated three cholinergic biomarkers at the end of the period of exposure: First, we assessed nAChR binding with [^{3}H]cytisine, a ligand that binds selectively to the $\alpha4\beta2$ nAChR, the predominant subtype in mammalian brain that is upregulated by nicotine (Abreu-Villaça et al., 2003a; Flores et al., 1992; Happe et al., 1994; Whiting and Lindstrom, 1987) and, in some brain regions, by ethanol (Booker and Collins, 1997) administration. We also assessed choline acetyltransferase activity (ChAT) and the binding of [^{3}H]hemicholinium-3 (HC-3) to the high-affinity presynaptic choline transporter. ChAT, the enzyme responsible for acetylcholine biosynthesis, is a constitutive marker for cholinergic nerve terminals and serves as an archetypal measure of cholinergic innervation, but its activity does not respond to changes in impulse flow. As a result, ChAT reflects the concentration of cholinergic nerve terminals (Aubert et al., 1996; Happe and Murrin, 1992; Navarro et al., 1989; Zahalka et al., 1992, 1993). In contrast, high-affinity choline uptake, as assessed by the binding of HC-3 to the presynaptic high-affinity choline transporter, is responsive to neuronal activity (Klemm and Kuhar, 1979; Simon et al., 1976) and the comparative changes in ChAT and HC-3 binding allows for the distinction between effects on the concentration of synaptic terminals and effects on synaptic activity (Aubert et al., 1996; Happe and Murrin, 1992; Navarro et al., 1989; Zahalka et al., 1992, 1993).

2. Results

2.1. *Effects on fluid intake/body weight, body weight and brain region weight*

Fluid intake/body weight (day: $F=23.3$; $df=8.6$; $P<0.001$) and body weight (day: $F=205.4$, $df=2.5$; $P<0.001$) significantly

Table 1 – Mean fluid intake/body weights, body weights and brain region weights

Measure	VEH	ETOH	NIC	NIC+ETOH
Fluid intake/ body weight [a]	0.32±0.006	0.30±0.005	0.29±0.005	0.32±0.005
Body weight [b]				
Male	12.1±0.3	12.9±0.2	13.1±0.2	13.1±0.2
Female	12.1±0.3	11.4±0.2	11.2±0.2	11.2±0.2
Brain region weight [c]				
Cerebral cortex	93.6±1.9	90.9±1.5	91.9±1.7	91.6±1.1
Midbrain	63.2±1.0	63.8±1.0	64.0±1.2	63.6±1.0

Daily measurements from treatment period (PN30 to PN45) were used to calculate mean values of fluid intake/body weights and body weights.
[a] Milliliters/gram.
[b] Grams.
[c] Milligrams.

Table 2 – Control values for cholinergic biomarkers

Measure	Cerebral cortex		Midbrain	
	Male	Female	Male	Female
nAChR binding (fmol/mg)	27.5±1.8	33.0±1.7 *	46.7±5.1	38.1±1.7
HC-3 binding (fmol/mg)	42.1±1.6	40.0±2.5	17.3±0.5	18.1±0.7
ChAT activity (pmol/mg/min)	1318±65	1479±45	1132±23	1146±36

The asterisks indicate control values for which females differ significantly from males.
* $P<0.001$.

increased throughout the exposure period. However, the rANOVAs on these variables did not indicate significant Treatment effects or interactions. Males tended to be heavier than females, however the effect only approached significance ($F=2.3$, $df=2.5$; $P=0.09$). Regarding brain region weights, the rANOVA did not indicate any significant effects of Treatment, Sex or interaction between these two factors. The mean values for these variables are presented in Table 1.

As expected, daily nicotine consumption in the NIC and NIC+ETOH groups varied from animal to animal. The distributions of consumption data were clearly normal for both groups (NIC K–S: $Z=0.5$; $P>0.1$; NIC+ETOH K–S: $Z=0.3$; $P>0.1$). The analysis of the Pearson's correlation coefficients indicated that the variations in the amount of nicotine that the animals (NIC and NIC+ETOH groups) consumed were not associated with any of the cholinergic biomarkers ($P>0.1$ in all cases).

2.2. *Overall analysis of cholinergic biomarkers*

The global rANOVA, using the one-dimensional design (four levels: VEH, ETOH, NIC and NIC+ETOH), across all cholinergic biomarkers, treatments, brain regions and sexes identified a significant interaction of Treatment×Cholinergic Biomarker ($F=4.0$, $df=6$; $P<0.001$). With the Ethanol and Nicotine treatments considered as separate factors in a two-dimensional design, we again found interactions indicative of selective effects on each biomarker: Nicotine×Cholinergic Biomarker ($F=7.3$, $df=2$; $P<0.001$). In addition, the two dimensions (Ethanol and Nicotine treatments) interacted with each other and with cholinergic biomarker and region: Ethanol×Nicotine×Cholinergic Biomarker ($F=4.7$, $df=2$; $P=0.01$), Ethanol×Nicotine×Region ($F=3.1$, $df=1$; $P=0.08$), Ethanol×Nicotine×Cholinergic Biomarker×Region ($F=3.0$, $df=2$; $P=0.05$). Given these results, data analysis was carried out separately on each biochemical marker and brain region and then the results were reexamined. Treatments were also interactive with sex: Ethanol×Nicotine×Sex ($F=4.3$, $df=1$; $P=0.04$) and Ethanol× Nicotine×Cholinergic Biomarker×Region×Sex ($F=2.4$, $df=2$; $P=0.09$). So, after subdividing the data, we kept this factor in the analysis to see whether treatment interactions remained detectable.

To facilitate comparisons across multiple tissues and variables, the effects of each treatment on cholinergic biomarkers are given as the percent change from the corresponding control group, but statistical comparisons were made on raw data. For reference, control values appear in Table 2.

2.3. *Effects on nAChRs ([3H]cytisine binding)*

In the cerebral cortex, the one-dimensional ANOVA indicated a main Treatment effect ($F=5.3$; $df=3$; $P=0.003$). Post hoc

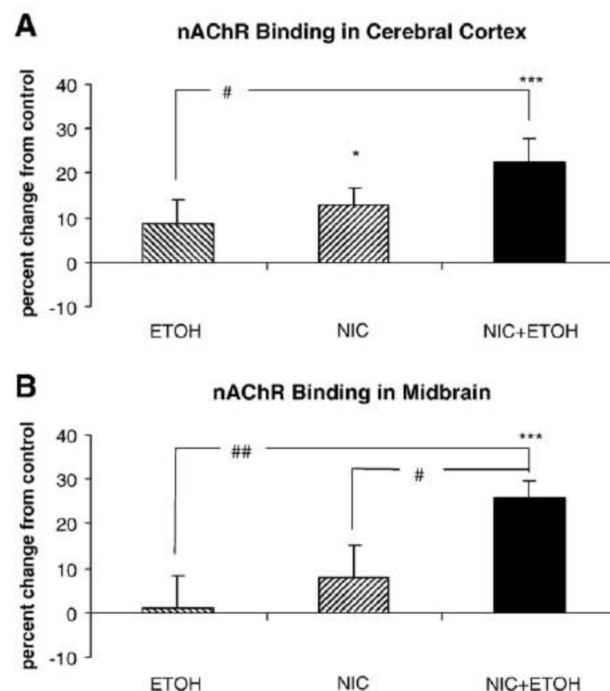

Fig. 1 – Effects of adolescent nicotine and/or ethanol on nAChR binding, presented as the percent change from control values (see Table 1): (A) in the cerebral cortex and (B) in the midbrain. Asterisks indicate differences from control group (*P<0.05; ***P<0.001) and # indicate differences between the other groups (#P<0.05; ##P<0.01), significant differences between groups as revealed by FPLSD. Values are means±SEM. NIC, nicotine exposure group; ETOH, ethanol exposure group; NIC+ETOH, nicotine and ethanol exposure group.

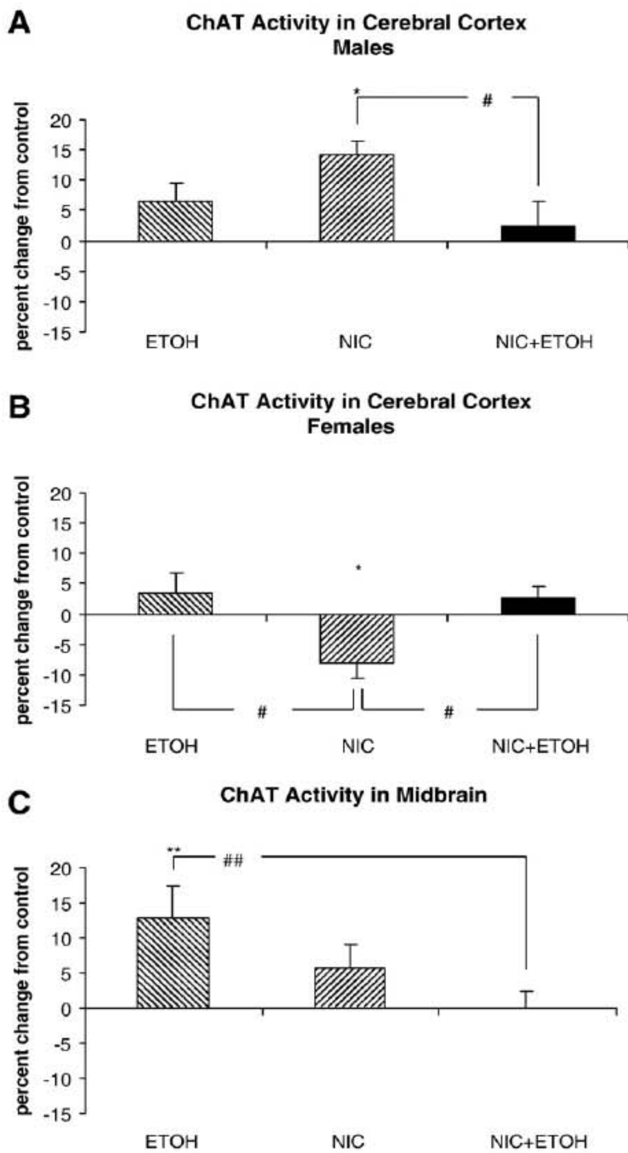

Fig. 2 – Effects of adolescent nicotine and/or ethanol on ChAT activity, presented as the percent change from control values. In cerebral cortex, the ANOVA indicated a Sex × Treatment interaction. Therefore, male (A) and female (B) results are presented separately. (C) ChAT activity in midbrain. Asterisks indicate differences from control group (*P < 0.05; **P < 0.01) and # indicate differences between the other groups (#P < 0.05; ##P < 0.01), significant difference between groups as revealed by FPLSD. Values are means ± SEM. NIC, nicotine exposure group; ETOH, ethanol exposure group; NIC + ETOH, nicotine and ethanol exposure group.

analyses (Fig. 1A) indicated that adolescent ethanol exposure by itself had little effect on nAChRs (ETOH > VEH; 9%). In contrast, nicotine administration produced a more robust nAChR upregulation (FPLSD: NIC > VEH: 13%; $P = 0.04$) while the combined NIC + ETOH treatment elicited an even more intense (FPLSD: NIC + ETOH > VEH: 22%; $P < 0.001$) effect. The difference between NIC + ETOH and ETOH groups also reached significance (FPLSD: NIC + ETOH > ETOH; $P = 0.04$). Using the two-dimensional design (Ethanol and Nicotine treatments considered as separate factors), the ANOVA did not show significant interactions between the nicotine and ethanol, indicating a simple summation of nicotine and ethanol effects.

In the midbrain, the one-dimensional ANOVA also indicated a Treatment effect ($F = 5.3$; $df = 3$; $P = 0.003$). Post hoc analyses (Fig. 1B) indicated that adolescent ethanol exposure by itself had little or no effect on nAChRs (ETOH > VEH; 1%). In addition, nicotine administration also only produced a modest nAChR upregulation (NIC > VEH; 8%) that failed to reach statistical significance. The NIC + ETOH treatment, however, elicited a robust nAChR upregulation (FPLSD: NIC + ETOH > VEH; 26%): In fact, significant differences were identified between NIC + ETOH and all other groups (FPLSD: NIC + ETOH > VEH; $P = 0.001$. NIC + ETOH > NIC; $P = 0.02$. NIC + ETOH > ETOH; $P = 0.002$). Expanding the analysis to consider Ethanol and Nicotine treatments as two separable factors, we identified a significant interaction between these factors, which indicated that the effect of combined nicotine and ethanol treatment could not be statistically accounted for by the summation of the two individual sets of effects. Although neither nicotine nor ethanol alone elicited significant nAChR upregulation when compared to the VEH group, there was a significant effect due to the combined exposure, revealing a synergistic effect of Nicotine and Ethanol.

2.4. *Effects on ChAT activity*

In the cerebral cortex, with Treatment considered as a one-dimensional factor, the ANOVA detected a significant interaction between Treatment and Sex ($F = 5.4$, $df = 3$; $P = 0.003$). After separation by sex, we found significant effects for both males and females. Interestingly, treatment effects differed between sexes. For male mice (Fig. 2A), the ANOVA indicated a main Treatment effect ($F = 3.0$, $df = 3$; $P > 0{,}05$). Nicotine exposure alone elicited an increase in ChAT activity (FPLSD: NIC > VEH; $P = 0.01$). However, ethanol treatment reversed the nicotine-induced increase in ChAT, as indicated by the significant difference between values for the nicotine and combined exposure groups (FPLSD: NIC > NIC + ETOH; $P = 0.03$) and by the less-than-additive effect of Nicotine and Ethanol indicated by the Nicotine × Ethanol interaction in the two-dimensional design ($F = 6.0$, $df = 1$; $P = 0.02$). For female mice (Fig. 2B), the ANOVA also detected a Treatment effect ($F = 3.2$, $df = 3$; $P = 0.04$). However, this effect was caused by the fact that female mice exposed to nicotine presented decreased ChAT activity when compared to all others groups (FPLSD: NIC < VEH; $P = 0.04$. NIC < ETOH; $P = 0.02$. NIC < NIC + ETOH; $P = 0.01$). In this case, the two-dimensional analysis failed to find a significant Nicotine × Ethanol interaction, connoting the fact that the effect of combined exposure was undistinguishable from simple additivity of the effects of nicotine and ethanol.

In the midbrain (Fig. 2C), considering the four treatments in a one-dimensional design, the ANOVA detected a Treatment effect ($F = 4.7$, $df = 3$; $P = 0.006$). The ETOH group presented increased ChAT activity when compared to the VEH (FPLSD: $P = 0.005$) and NIC + ETOH (FPLSD: $P = 0.005$) groups. The two-dimensional analysis found a significant Nicotine × Ethanol interaction ($F = 9.7$, $df = 1$; $P = 0{,}003$); indicative of less-than-additive effects of Nicotine and Ethanol.

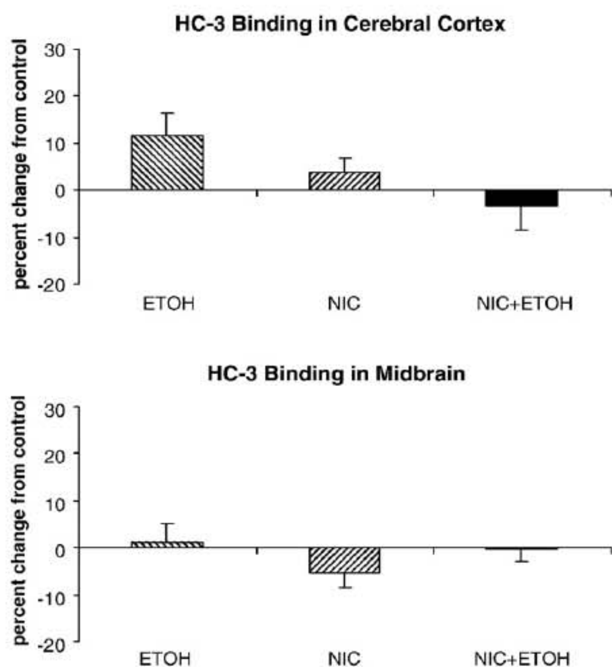

Fig. 3 – Effects of adolescent nicotine and/or ethanol on [³H] HC-3 binding, presented as the percent change from control values: (A) in the cerebral cortex and (B) in the midbrain. Values are means ± SEM. NIC, nicotine exposure group; ETOH, ethanol exposure group; NIC + ETOH, nicotine and ethanol exposure group.

2.5. *Effects on choline transporter ([3H]HC-3 binding)*

In both brain regions, the ANOVA failed to indicate significant effects of Treatment (Fig. 3) or Sex. The interaction between these two factors also did not reach statistical significance.

3. Discussion

Tobacco smoking and alcohol consumption are frequently associated during adolescence (Chen and Kandel, 1995; Torabi et al., 1993; Schorling et al., 1994). In spite of the epidemiological data, little is known about the neurobiological substrates that may underlie this association, particularly at the critical developmental period of adolescence. The present study provides experimental evidence that nicotine and ethanol have interacting effects on the central cholinergic system during adolescence.

3.1. *Methodological issues*

In the present study, C57BL/6 mice were chosen due to the fact that adult and periadolescent mice from this strain tolerate the bitter taste of nicotine in the concentration used here (Abreu-Villaça et al., 2006, 2007, 2008; Klein et al., 2003, 2004; Sparks and Pauly, 1999). In fact, our results did not indicate any difference in fluid consumption between groups. Another particular characteristic of this strain is the higher voluntary ethanol consumption (Crawley et al., 1997), which should be taken into consideration in studies that involve the administration of this substance, even though the bases underlying this strain-specific characteristic remain poorly understood (Crabbe et al., 1999). As a result, generalizations should be made with care and other strains and species may provide valuable data in future studies of ethanol and nicotine interactions.

The nicotine concentration used in the drinking solution has been shown to generate cotinine (main metabolite of nicotine) plasma levels (Klein et al., 2003, 2004; Sparks and Pauly, 1999) that are within the range of those found in adolescent smokers (Caraballo et al., 2004; Wood et al. 2004). Specifically, a previous study showed that exposure of C57BL/6 mice to 50 μg/ml nicotine in the drinking water elicits cotinine plasma levels of 50 ng/ml (Sparks and Pauly 1999). As for ethanol, according to previous studies in adolescent rodents, even the moderate dose used in the present study results in blood levels well above the legal driving limit (Silveri and Spear, 2000). Effects of the combined exposure could be due to nicotine and ethanol pharmacokinetic interactions. In this regard, it has been demonstrated that ethanol plasma levels are reduced in animals concomitantly exposed to nicotine (Chen and Harle, 2005; Gilbertson and Barron, 2005). However, this effect was only described in animals that received oral ethanol, and seemed to be due to a nicotine-induced delay in gastric emptying (Chen and Harle, 2005; Scott et al., 1993), leading to an increased ethanol metabolization by the gastric alcohol dehydrogenase (Oneta et al., 1998). In addition, it has been demonstrated that i.p. ethanol concentrations are not affected by chronic nicotine exposure (Parnell et al., 2006) and that nicotine exposure does not affect the elimination rate of ethanol (Collins et al., 1988). Finally, nicotine plasma levels and the elimination rate of nicotine do not seem to be affected by ethanol exposure (Collins et al., 1988). These findings suggest that metabolic nicotine–ethanol interactions are not capable of explaining our current findings.

In the present study, we used a single ligand concentration that lies above the Kd but nevertheless below full saturation of the binding site for determinations of nicotinic receptor subtype and presynaptic choline transporter binding. The strategy of using a single, subsaturating ligand concentration enables the detection of drug-induced changes regardless of whether the changes are in affinity (Kd) or capacity (B_{max}), but does not distinguish between the two mechanisms. The interpretation of results of the present study does not depend upon whether the change is specific to concentration or affinity. However, previous studies demonstrated that nicotine and ethanol affect mainly the concentration of nAChRs and HC-3 binding sites (Dohrman and Reiter, 2003; Trauth et al., 1999; Zahalka et al., 1993).

3.2. *Effects on nAChRs*

It has been consistently demonstrated that nicotine exposure elicits nAChR upregulation in the adult rat brain (Doura et al., 2008; Flores et al., 1992). Previous studies have also demonstrated that adolescent rats exposed to nicotine present more robust and persistent upregulation as compared to adults (Abreu-Villaça et al., 2003a; Doura et al., 2008; Trauth et al.,

1999). In general, the present results confirm previous findings in adolescent rodents: nicotine elicited a significant nAChR upregulation in the cerebral cortex. However, in the midbrain, nicotine administration only produced a modest increase in [3H]cytisine binding. Differences in the magnitude of effects between studies could be due to differences in experimental design regarding exposure procedures. Most studies that described the more robust nAChR upregulation used continuous nicotine infusions (Abreu-Villaça et al., 2003a). In contrast, we chose to give animals free access to a nicotine solution in the drinking water, a model that mimics the human pattern of intermittent exposure and allows for consumption during the active period of the mice. In fact, a previous study indicated that continuous exposure results in a more consistent upregulation when compared to intermittent one (Abreu-Villaça et al., 2003a), which may allow for some recovery in between doses. In addition, it is expected that the intensity of nicotine-induced upregulation varies between brain regions (Abreu-Villaça et al., 2003a) and cell types (Gaimarri et al., 2007; Nashmi et al., 2007). The present results indicate that oral nicotine in the concentration used here is pharmacologically active, which is in accordance with recent findings form our group describing that oral nicotine is effective in eliciting behavioral effects (Abreu-Villaça et al., 2006, 2007, 2008). Neurochemical alterations including nicotinic receptors upregulation (Nuutinen et al., 2005; Sparks and Pauly, 1999), altered monoamine levels and metabolism (Gaddnas et al., 2000; Tammimaki et al., 2006; Vihavainen et al., 2006), as well as altered expression of genes implicated in synaptic plasticity induced by drugs of abuse (Marttila et al., 2006) have been described previously, but only when higher doses were used.

It is well documented that ethanol directly affects the function of various ligand-gated ion channels, including nAChRs. In this regard, it was demonstrated that ethanol enhancement of dopamine release in the nucleus accumbens (Blomqvist et al., 1993) and ethanol-induced locomotor activity (Blomqvist et al., 1992) involve nAChR activation. Mecamylamine administration (nicotinic antagonist) reduces ethanol consumption in rats and a subchronically low dose of nicotine increases voluntary ethanol intake (Blomqvist et al., 1996). Cardoso et al. (1999), using different nAChR subtype compositions expressed in *Xenopus* oocytes, found that ethanol potentiates acetylcholine-induced currents in several nAChR subtypes including $\alpha4\beta2$, but has little effect on other subtypes. In addition, some studies demonstrated that ethanol inhibits $\alpha7$ nAChRs in oocytes (Breese et al., 1993; de Fiebre and de Fiebre, 2005; Yu et al., 1996). Regarding nAChRs binding, several studies have demonstrated that ethanol exposure may produce both upregulation and downregulation depending on the brain region or the cell line used (Booker and Collins, 1997; Gorbounova et al., 1998; Robles and Sabriá, 2008). In our study, ethanol exposure failed to promote significant alterations in nAChR binding. The short-term treatment and moderate dose used could explain the absence of ethanol effects. For instance, in vivo studies using short-term treatment did not detect an effect of ethanol in mice brain nAChRs (Burch et al., 1988; de Fiebre and Collins, 1993).

Interestingly, for both brain regions, the combined nicotine+ethanol exposure elicited a robust nAChR upregulation. In the cerebral cortex, this outcome reflected the summation of nicotine and ethanol effects. However, in the midbrain, despite the lack of effect elicited by either drug, nicotine+ethanol-elicited upregulation revealed a synergistic effect of nicotine and ethanol. It has been suggested that ethanol stabilizes the open state of the *Torpedo* nAChR (Wu et al., 1993) and may increase agonist affinity for this receptor (Forman et al., 1989). Dohrman and Reiter (2003) demonstrated that ethanol can modulate the nicotine-induced nAChRs upregulation in cell cultures. In general, studies in vivo suggest that ethanol may enhance the electrophysiological response to nicotine in some but not all brain areas due possibly to variations in the distribution of nAChR subtypes (Breese et al., 1993). In addition, concomitant nicotine and ethanol exposure in adult rats results in additive dopamine release in the nucleus accumbens, an effect that seems to be mediated by nAChR (Tizabi et al., 2007). Altogether, these findings indicate that ethanol may serve as a co-agonist to acetylcholine at some areas of the brain and that the co-exposure could generate a greater susceptibility to trigger a nAChR desensitization and, consequent upregulation. Finally, to our knowledge, our results in the midbrain constitute the first experimental evidence that the co-administration does have more than a simple additive effect in nAChR binding. Given the critical role of the midbrain circuitry in mechanisms of addiction (Mansvelder and McGehee, 2002; Nestler, 2001) and the fact that nAChR upregulation is hypothesized to play a role in these mechanisms (Buisson and Bertrand, 2002; Dani and De Biasi, 2001; Picciotto et al., 2008), we suggest that, if a similar effect occurs during human adolescents concomitant consumption of tobacco and ethanol, the synergistic effect may facilitate the development of abuse to these drugs.

The lack of synergistic effects in the cortex is, to some extent, puzzling since we were studying the same receptor subtype in both brain regions. It should be mentioned that nicotine regulates the release of several neurotransmitters including dopamine, serotonin, noradrenalin, glutamate, GABA and acetylcholine (Wonnacott, 1997). Besides its actions at nAChRs, ethanol has been shown to enhance function at GABA and glycine receptors, act as a co-agonist at serotonin receptors, and act as a functional antagonist at glutamate receptors (Larsson and Engel, 2004). Therefore, there are several other potential targets and, in fact, recent evidences point out that nicotine and ethanol interact affecting other neurotransmitter systems (Al-Rejaie and Dar, 2006a,b; Inoue et al., 2007). These indicate that additional molecular mechanisms, region-specific, may also play a role on nicotine and ethanol interactions. The role of other neurotransmitter systems and the integration of the effects, at synaptic and neural circuit levels, may explain differences in results between brain regions. These integrative effects, certainly relevant to the understanding of the mechanisms that underlie nicotine and ethanol adolescent co-use and abuse, remain unclear and need to be tested in subsequent studies.

3.3. *Effects on ChAT activity and HC-3 binding*

3.3.1. *ChAT activity*

The effects on ChAT differed substantially between brain regions. In the cerebral cortex, nicotine exposure generated a

sex-dependent effect with decreased ChAT activity for females and increased activity for males. ChAT is a constitutive cholinergic synaptic biomarker (Aubert et al., 1996; Happe and Murrin, 1992; Navarro et al., 1989; Zahalka et al., 1992, 1993) for which a decrease in activity indicates loss of cholinergic innervation and possibly loss of neural cells (Trauth et al., 2000a,b,c). In fact, while the activation of nAChRs serves a trophic role in neurodevelopment (Coronas et al., 2000; Hohmann and Berger-Sweeney, 1998; Navarro et al., 1989; Pugh and Margiotta, 2000), excessive cholinergic stimulation disrupts patterns of cell replication, differentiation and synaptogenesis (Levin and Slotkin, 1998; Slotkin, 2002, 2004), progressing at high levels to outright cell damage (Abreu-Villaça et al., 2003b; Abrous et al., 2002; Slotkin, 2002, 2004). Deficits in ChAT may reflect a higher susceptibility of females to negative effects of nicotine as compared to males. In this regard, adolescent female smokers show a more rapid onset of nicotine-dependence (DiFranza et al., 2002) and several animal studies suggest that females are more sensitive to cell damage due to adolescent nicotine exposure (Abreu-Villaça et al., 2003b; Trauth et al., 1999, 2000a, 2000b, 2000c). Alternatively, the elevations in ChAT detected in males suggest that nicotine exposure elicits a small but significant increase in the density of cholinergic innervation, which may represent sprouting as a compensatory response to cell damage, as suggested previously (Abreu-Villaça et al., 2003a, 2004a) or, in contrast, may be a response to the trophic effect of nAChRs activation (Coronas et al., 2000; Hohmann and Berger-Sweeney, 1998; Hohmann et al., 1988; Navarro et al., 1989; Pugh and Margiotta, 2000). In the midbrain, nicotine failed to elicit alterations in ChAT activity. Previous studies demonstrated that adolescent rats exposed to nicotine showed a decrease in cell number, which seems to include neurons and glial cells, and a compensatory increase in cell size (Abreu-Villaça et al., 2003b; Trauth et al., 2000a,b,c; Xu et al., 2003). Future studies will need to directly assess cell damage in order to confirm these results and verify whether it is restricted to cholinergic cells.

As for ethanol, we failed to find significant alterations in ChAT in the cerebral cortex while, in the midbrain, there was a small but significant increase in the density of cholinergic innervation. Previous studies have shown that intake of ethanol results in a significant reduction in ChAT (Arendt et al., 1988; Floyd et al., 1997), however, this effect was associated with prolonged periods of exposure. We suggest that a moderate dose of ethanol administrated every other day during a short period (as in the present study) does not have a consistent effect in the cerebral cortex. Considering the compensatory pattern identified in the midbrain, a degree of recovery in ChAT activity is also conceivable.

To our knowledge, this is the first study that describes the effects of the combined nicotine+ethanol exposure in ChAT activity. Surprisingly, we found that the co-administration failed to produce alterations in ChAT in either region examined. In the cerebral cortex, during female exposure, despite nicotine-elicited reduction in ChAT activity, exposure to both nicotine and ethanol failed to elicit cholinergic cell damage, which suggests a protective effect of ethanol. In contrast, for males, nicotine elicited elevations in ChAT. However, ethanol co-exposure reverted this effect. Finally, in the midbrain, the effect of nicotine+ethanol exposure was reduced when compared to either nicotine or ethanol exposure, being essentially similar to the vehicle group. The protective effect of ethanol was somewhat unexpected. Co-administration elicited stronger upregulation of nAChR when compared to either nicotine or ethanol exposure, and, as mentioned before, excessive cholinergic stimulation disrupts the normal pattern of neural cell development (Levin and Slotkin, 1998; Slotkin, 2002, 2004). In the present study, we did not evaluate whether nAChR upregulation reflects increased function. In fact, studies addressing this issue are still controversial (Gaimarri et al., 2007; Gentry and Lukas, 2002; Nashmi et al., 2007). It is possible that upregulation in part reflects a compensatory response to ethanol-elicited receptor desensitization, and, if so, the protective effect of ethanol could be due to the trophic effect of nAChRs activation associated with moderate activation of receptors (Coronas et al., 2000; Hohmann and Berger-Sweeney, 1998; Hohmann et al., 1988; Navarro et al., 1989; Pugh and Margiotta, 2000). Indeed, previous papers addressing cellular neurotoxicity of nicotine and/or ethanol have demonstrated less-than-additive effects (Penland et al., 2001; Tizabi et al., 2003, 2004, 2005). In contrast, a morphological study indicated that both nicotine and ethanol adolescent exposure elicit cell death in the dentate gyrus of rats and that co-administration may elicit a more potent effect when compared to either nicotine or ethanol exposure (Jang et al., 2002a). Altogether, these results indicate that even though nicotine+ethanol co-administration does not elicit significant damage to the cholinergic terminals, other cell types may suffer damage. Future studies addressing which brain regions and cell types are affected may help explain the co-use and abuse of these drugs as well as the associated behavioral alterations.

3.3.2. *HC-3 binding*

The high-affinity choline uptake system, assessed by HC-3 binding, is regulated by nerve impulse activity (Klemm and Kuhar, 1979; Simon et al., 1976). Regarding nicotine, decrements in HC-3 binding were expected since it has been previously demonstrated that adolescent treatment is effective in producing this effect across several brain regions (Abreu-Villaça et al., 2003a). Conversely, our findings failed to show effects of nicotine in HC-3 binding. Divergent results may be explained by differences inherent to the various routes of administration that have been used. In contrast to other routes such as osmotic minipumps (which produce constant nicotine plasma levels) or injections (which allow for a precise timing of sacrifice relative to the injection) (Abreu-Villaça et al., 2003a), oral nicotine mimics intermittent exposure characteristic of smokers. Previous reports indicate that HC-3 binding responds within minutes to the changes in impulse activity (Ford et al., 1999; Happe and Murrin, 1992), therefore, inter-individual differences in the time interval between the last drinking episode and sacrifice may be the most likely reason for the absence of effects concerning this cholinergic marker.

As for ethanol, despite methodological differences between studies, the lack of effect described in the present study is in line with previous reports that describe that the high-affinity choline uptake system is highly resistant to

ethanol effects (Saltarelli et al., 1990; Kristofiková et al., 2003). This resistance has been interpreted based on the assumption that the effects of ethanol are basically due to its detergent actions on membrane lipids and, consequently, on membrane-bound enzymes such as Na+/K+-ATPase, whose activity is directly linked to the function of high-affinity systems (Sun and Sun, 1985). These studies demonstrate that only high doses of ethanol are effective in eliciting alterations.

The combined exposure also failed to produce alterations in HC-3 binding in the present work. Altogether, these results suggest that with our experimental design, nicotine, ethanol and the combined exposure during adolescence are not capable of eliciting alterations in cholinergic synaptic activity. The comparative changes in ChAT activity and HC-3 binding permit the distinction between effects on synaptic outgrowth as distinct from synaptic activity (Aubert et al., 1996; Happe and Murrin, 1992; Navarro et al., 1989; Zahalka et al., 1992, 1993). Adolescent nicotine treatment produced effects on ChAT that were separable from HC-3 binding results. In fact, the lack of alterations in HC-3 binding indicates that the effects of nicotine and/or ethanol exposure largely reflect altered cholinergic innervation.

3.4. *Conclusions*

These results indicate that the central cholinergic system is a site at which nicotine and ethanol interact. This interaction may underlie the association between tobacco and alcohol consumption during adolescence. Adolescent tobacco and ethanol consumption are severe public health issues. Brain development continues into adolescence, being characterized by several modifications (Altman and Bayer, 1990; Rakic et al., 1994), such as the maturation of the central cholinergic system (Zahalka et al., 1993). In this regard, recent studies from our group provide experimental evidence for functional interactions between nicotine and ethanol in the regulation of memory/learning and anxiety behavioral responses during adolescence (Abreu-Villaça et al., 2007, 2008). Considering the role of the cholinergic system in both memory/learning and anxiety (Dani and Bertrand, 2007; Degroot and Treit, 2002; Engin and Treit, 2007; File et al., 2000; Hasselmo, 2006; Ricceri et al., 2002; Viveros et al., 2007), the behavioral effects of nicotine+ethanol support the assumption that the cholinergic system is a site at which these drugs interact. The understanding of the neurochemical interactions of drugs may be essential for the development of new pharmacological therapies capable of reversing the addictive state, preventing relapse and/or reducing the intake of these drugs. The present study provides direct experimental evidence for neurochemical interactions between nicotine and ethanol, which may explain, at least in part, the frequent association between tobacco and alcohol consumption during adolescence. Particularly, the concomitant exposure elicited both less-than-additive and synergistic interactions between the two treatments in the central cholinergic system: Upregulation of nAChR reflected synergistic effects of nicotine and ethanol in the midbrain, a region associated with mechanisms of reward and addiction (Mansvelder and McGehee, 2002; Nestler, 2001). Additionally, alterations in ChAT activity elicited by either drug were reduced by co-exposure.

The lack of effects of nicotine+ethanol exposure in cholinergic innervation and activity indicates that direct effects on cholinergic synaptic function cannot explain the highly frequent concomitant consumption during adolescence (Chen and Kandel, 1995; Torabi et al., 1993; Schorling et al., 1994) and the behavioral alterations described in animal models (Abreu-Villaça et al., 2007, 2008). However, these effects may be determined, at least in part, by the peculiar effects of the combined exposure on nAChR, either directly or mediated by the activation of other neurotransmitter systems. Particularly, considering that nAChR is hypothesized to play a role in addiction mechanisms (Buisson and Bertrand, 2002; Dani and De Biasi, 2001), the more robust nAChR upregulation identified in the nicotine+ethanol group suggests that, if a similar effect occurs during adolescents' concomitant consumption of tobacco and ethanol, individuals in this age group are more prone to become addicted to these drugs.

4. Experimental procedures

4.1. *Animal treatment*

All experiments were carried out in accordance with the declaration of Helsinki and with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health. C57BL/6 mice were chosen because prior reports demonstrated that adult and periadolescent mice from this strain consume nicotine in the concentration used in the present study (Abreu-Villaça et al., 2006, 2007, 2008; Klein et al., 2003, 2004; Sparks and Pauly, 1999). All mice were bred and maintained in our laboratory. Animals were derived from a C57BL/6 colony maintained at the Universidade Estadual de Campinas (São Paulo, Brazil) for over 70 generations. The animals were kept in a temperature-controlled room on a 12 h light/dark cycle (lights on at 2:00 am). Access to food and water was *ad lib*. On the first postnatal day (PN1), litters were culled to a maximum of 8 mice to ensure standard nutrition. At weaning (PN25) animals were separated by sex and allowed free access to food and water.

On PN29, pups from 14 litters (33 females and 39 males) were individually housed which allowed for accurate individual measurement of fluid intake/nicotine consumption. Animals were exposed to nicotine and/or ethanol from PN30 to PN45, the approximate age range during which animals of both genders and most breeding stock exhibit adolescent-typical behavioral characteristics and particular neurochemical and endocrine patterns when compared to adulthood and pre-pubertal periods (Spear, 2000a,b). During this period, 25% ethanol (2 g/kg) solution (v/v) in saline or saline only was injected (i.p.) every other day in order to mimic cyclical patterns of alcohol consumption; while (−)-nicotine free base (50 μg/ml) in 2% saccharin or 2% saccharin only was administered in the drinking water (the sole source of fluid) in order to mimic intermittent nicotine consumption. Each one of the following treatment groups was composed of no more than one male and one female mice from each litter:

VEH (oral saccharin+injected saline), ETOH (oral saccharin +injected ethanol), NIC (oral nicotine/saccharin+injected saline) and those receiving the combined treatment: NIC +ETOH (oral nicotine/saccharin+injected ethanol). Bottles were cleaned and refilled daily. Loss due to leakage was measured from a bottle placed in an empty cage ("blank") and subtracted from fluid consumption data. Body weights and fluid consumption were also measured every day. Since body weight increases significantly during adolescence, daily fluid intake data were obtained by dividing the absolute values of fluid intake of each animal by its own body weight.

Studies were conducted at the end of the drug administration period (PN45). Animals were decapitated during their dark cycle and the cerebral cortex and midbrain were dissected (Trauth et al., 2000a,b,c), frozen in liquid nitrogen, and stored at −45 °C until assayed. For each treatment group, 15–19 animals were examined, equally divided into males and females.

4.2. *Tissue preparation and assays*

Tissues were thawed and homogenized (Ultra-Turrax T10 basic, IKA, São Paulo, SP) in 40 volumes of ice-cold 50 mM Tris HCl (pH 7.4) and aliquots were withdrawn for measurements of ChAT activity (Lau et al., 1988) and total protein (Bicinchoninic Acid kit) (Smith et al., 1985). To prepare the cell membrane fraction, the homogenates were sedimented at 40,000×*g* for 10 min and the supernatant solution was discarded. The membrane pellet was resuspended (Ultra-Turrax) in the original volume of buffer, resedimented, and the resultant pellet was resuspended in approximately 10 volumes (based on the original weight of the tissue) of the same buffer using a smooth glass homogenizer fitted with a Teflon pestle. Aliquots of this resuspension were withdrawn for measurements of [^{3}H]cytisine and [^{3}H]HC-3 binding, and for membrane protein (Bicinchoninic Acid kit).

All assays have been described in detail in previous papers (Abreu-Villaça et al., 2003a, 2004a; Trauth et al., 1999, 2000a, 2000b, 2000c) and will therefore be presented briefly. ChAT activity was assayed in tissue homogenate using 50 μM [^{14}C] acetyl-coenzyme A. Labeled acetylcholine was then extracted and the activity was determined relative to tissue protein. [^{3}H] Cytisine and [^{3}H]HC-3 binding were determined in the cell membrane fraction using final ligand concentrations of 2 nM; specific binding was displaced with 100 μM nicotine for nAChRs and 20 μM unlabeled HC-3 for the high-affinity choline transporter. Values were then calculated as specific binding per mg of membrane protein.

4.3. *Data analysis of the effects on fluid intake/body weight, body weight and brain region weight*

Initially, repeated-measures analyses of variance (rANOVA) on each variable (fluid intake/body weight and body weight) were carried out. Treatment (VEH, ETOH, NIC and NIC+ETOH) and Sex were used as between-subjects factors. Day was considered the within-subjects factor. Whenever significant Treatment×Day interactions were detected, pairwise post-hoc analyses were carried out by using Fisher's Protected Least Significant Difference (FPLSD). Whenever significant Treatment×Sex interactions were detected, appropriate lower-order ANOVAs were utilized, followed by pairwise post-hoc analyses (FPLSD). Effects of nicotine and/or ethanol treatment on brain region weights were conducted with a separate ANOVA (nicotine and/or ethanol Treatment, Sex) for each brain region.

4.4. *Data analysis of cholinergic biomarkers*

To reduce the likelihood of type 1 statistical errors that might result from repeated testing of the global data set, we first performed a global rANOVA on all factors: Treatment (VEH, ETOH, NIC and NIC+ETOH), Brain Region and Sex, utilizing log-transformation because of the heterogeneous variance among the different biomarkers. The Cholinergic Biomarkers (nAChR binding, ChAT, HC-3 binding) were treated as repeated measures, since each was obtained from the same tissue homogenate. Whenever this initial test indicated treatment effects that differed among different biomarkers and brain regions, data were then examined separately, using ANOVAs [Treatment (VEH, NIC, ETOH and NIC+ETOH) and Sex as between-subjects factors]. Whenever significant Treatment×Sex interactions were detected, appropriate lower-order ANOVAs were utilized, followed by pairwise post-hoc analyses (FPLSD). For all cases in which treatment effects did not interact with other variables, only the main effect was recorded, without testing of individual differences.

4.5. *Data analysis of nicotine–ethanol interactions*

The one-dimensional statistical design described above, in which Treatment (VEH, ETOH, NIC and NIC+ETOH) was considered the between-subjects factor, was used to verify whether significant differences between the four treatment groups existed. However, the combined use of nicotine and ethanol can have more-than-additive (synergistic), less-than-additive or additive effects which are not addressed by the one-dimensional design. Therefore, a two-dimensional design was also used (Abreu-Villaça et al., 2004a,b, 2007, 2008; Rhodes et al., 2003). In this design Ethanol (treated: ETOH and NIC+ETOH; non-treated: VEH and NIC) was considered one of the between-subjects factors. Nicotine, (treated: NIC and NIC+ETOH; non-treated: VEH and ETOH) was considered the other between-subjects factor. In this formulation, more-than-additive (synergistic) and less-than-additive effects appear as significant interactions between the two treatment dimensions, whereas simple, additive effects do not show significant interactions.

4.6. *Association between nicotine and cholinergic biomarkers*

Considering that animals had *ad lib.* access to the nicotine solution, variations in the amount of nicotine received were to be expected. The normality of the distributions of average daily nicotine consumption per animal was assessed by means of Kolmogorov–Smirnov one sample tests (K–S). In order to verify whether there were significant associations between the total amount of nicotine received and the biochemical biomarkers, Pearson's correlation coefficients (r)

were calculated. The analysis was carried out initially using the entire data set and subsequently segmenting the data set into Treatment (NIC and NIC+ETOH) and Sex groups.

4.7. *General aspects of the data analysis*

Significance was assumed at the level of $P<0.05$ for main effects; however, for interactions at $P<0.1$, we also examined whether lower-order main effects were detectable after subdivision of the interactive variables (Snedecor and Cochran, 1967). Data are compiled as means and standard errors.

4.8. *Materials*

Radioisotopically-labeled compounds came from PerkinElmer Life Sciences (Boston, MA): [^{14}C]acetyl-CoA (specific activity 4 mCi/mmol), [^{3}H]HC-3 (specific activity 144.5 Ci/mmol) and [^{3}H]cytisine (specific activity, 40 Ci/mmol). Sigma Chemical Co. (St. Louis, MO) was the source for bovine albumin, BCA kit, eserine hemisulfate salt, 3-heptanone, (–)-nicotine hydrogen tartate salt, (–)-nicotine free base, sodium tetraphenylborate and triton X-100. VETEC Química Fina Ltda (Rio de Janeiro, RJ) was the source for all other reagents.

Acknowledgments

This work was supported by grants from Philip Morris USA Inc. and Philip Morris International, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-BRAZIL) and by fellowships from Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ-BRAZIL) and Sub-reitoria de Pós-graduação e Pesquisa da Universidade do Estado do Rio de Janeiro (SR2-UERJ). The authors are thankful to Armando Meyer for his helpful comments, to Theodore Slotkin and Frederick Seidler for their help with methodological issues and to Edson Oliveira for animal care.

REFERENCES

Abreu-Villaça, Y., Seidler, F.J., Qiao, D., Tate, C.A., Cousins, M.M., Thillai, I., Slotkin, T.A., 2003a. Short-term adolescent nicotine exposure has immediate and persistent effects on cholinergic systems: critical periods, patterns of exposure, dose thresholds. Neuropsychopharmacology 28, 1935–1949.

Abreu-Villaça, Y., Seidler, F.J., Tate, C.A., Slotkin, T.A., 2003b. Nicotine is a neurotoxin in the adolescent brain: critical periods, patterns of exposure, regional selectivity, and dose thresholds for macromolecular alterations. Brain Res. 979, 114–128.

Abreu-Villaça, Y., Seidler, F.J., Tate, C.A., Cousins, M.M., Slotkin, T.A., 2004a. Prenatal nicotine exposure alters the response to nicotine administration in adolescence: effects on cholinergic systems during exposure and withdrawal. Neuropsychopharmacology 29, 879–890.

Abreu-Villaça, Y., Seidler, F., Slotkin, T.A., 2004b. Does prenatal nicotine exposure sensitize the brain to nicotine-induced neurotoxicity in adolescence? Neuropsychopharmacology 29, 1440–1450.

Abreu-Villaça, Y., Queiroz-Gomes, F.E., Dal Monte, A.P., Filgueiras, C.C., Manhães, A.C., 2006. Individual differences in novelty-seeking behavior but not in anxiety response to a new environment can predict nicotine consumption in adolescent C57Bl/6 mice. Behav. Brain Res. 167, 175–182.

Abreu-Villaça, Y., Medeiros, A.H., Lima, C.S., Faria, F.P., Filgueiras, C.C., Manhães, A.C., 2007. Combined exposure to nicotine and ethanol in adolescent mice differentially affects memory and learning during exposure and withdrawal. Behav. Brain Res. 181, 136–146.

Abreu-Villaça, Y., Nunes, F., Queiroz-Gomes, F.E., Manhães, A.C., Filgueiras, C.C., 2008. Combined exposure to nicotine and ethanol in adolescent mice differentially affects anxiety levels during exposure, short term and long term withdrawal. Neuropsychopharmacology 33, 599–610.

Abrous, D.N., Adriani, W., Montaron, M.F., Aurousseau, C., Rougon, G., Le Moal, M., Piazza, P.V., 2002. Nicotine self-administration impairs hippocampal plasticity. J. Neurosci. 22, 3656–3662.

Aistrup, G.L., Marszalec, W., Narahashi, T., 1999. Ethanol modulation of nicotinic acetylcholine receptor currents in cultured cortical neurons. Mol. Pharmacol. 55, 39–49.

Altman, J., Bayer, S.A., 1990. Migration and distribution of two populations of hippocampal granule cell precursors during the perinatal and postnatal periods. J. Comp. Neurol. 301, 365–381.

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

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

Arendt, T., Hennig, D., Gray, J., Marchbanks, R., 1988. Loss of neurons in rat basal forebrain projection system after prolonged intake of ethanol. Brain Res. Bull. 21, 563–570.

Aubert, I., Cecyre, D., Gauthier, S., Quirion, R., 1996. Comparative ontogenic profile of cholinergic markers, including nicotinic and muscarinic receptors, in the rat brain. J. Comp. Neurol. 369, 31–55.

Batel, P., Pessione, F., Maitre, C., Rueff, B., 1995. Relationship between alcohol and tobacco dependencies among alcoholics who smoke. Addiction 90, 977–980.

Benowitz, N.L., 1992. Cigarette smoking and nicotine addiction. Med. Clin. North Am. 76, 415–437.

Blomqvist, O., Soderpalm, B., Engel, J.A., 1992. Ethanol-induced locomotor activity: involvement of central nicotinic acetylcholine receptors? Brain Res. Bull. 29, 173–178.

Blomqvist, O., Engel, J.A., Nissbrandt, H., Söderpalm, B., 1993. The mesolimbic dopamine-activating properties of ethanol are antagonized by mecamylamine. Eur. J. Pharmacol. 249, 207–213.

Blomqvist, O., Ericson, M., Johnson, D.H., Engel, J.A., Soderpalm, B., 1996. Voluntary ethanol intake in the rat: effects of nicotinic acetylcholine receptor blockade or subchronic nicotine treatment. Eur. J. Pharmacol. 314, 257–267.

Booker, T.K., Collins, A.C., 1997. Long-term ethanol treatment elicits changes in nicotinic receptor binding in only a few brain regions. Alcohol 14, 131–140.

Breese, G.R., Morrow, A.L., Simson, P.E., Criswell, H.E., McCown, T.J., Duncan, G.E., Keir, W.J., 1993. The neuroanatomical specificity of ethanol action on ligand-gated ion channels: a hypothesis. Alcohol Alcohol Suppl. 2, 309–313.

Buisson, B., Bertrand, D., 2002. Nicotine addiction: the possible role of functional upregulation. Trends Pharmacol. Sci. 23, 130–136.

Burch, J.B., de Fiebre, C.M., Marks, M., Collins, A.C., 1988. Chronic ethanol or nicotine treatment results in partial cross-tolerance between these agents. Psychopharmacology (Berlin) 95, 452–458.

Caraballo, R.S., Giovino, G.A., Pechacek, T.F., 2004. Self-reported cigarette smoking vs. serum cotinine among U.S. adolescents. Nicotine Tob. Res. 6, 19–25.

Cardoso, R.A., Brozowski, S.J., Chavez-Noriega, L.E., Harpold, M., Valenzuela, C.F., Harris, R.A., 1999. Effects of ethanol on recombinant human neuronal nicotinic acetylcholine

receptors expressed in *Xenopus* oocytes. J. Pharmacol. Exp. Ther. 289, 774–780.
Carmody, T.P., Brischetto, C.S., Matarazzo, J.D., O'Donnell, R.P., Connor, W.E., 1985. Co-occurrent use of cigarettes, alcohol, and coffee in healthy, community-living men and women. Health Psychol. 4, 323–335.
Centers for Disease Control and Prevention, 2000. Trends in cigarette smoking among high school students: United States, 1991–1999. Morbid. Mortal. Wkly. Rpt. 49, 755–758.
Chen, K., Kandel, D.B., 1995. The natural history of drug use from adolescence to the mid-thirties in a general population sample. Am. J. Public Health 85, 41–47.
Chen, W.J., Harle, L.K., 2005. Interactive effect of alcohol and nicotine on developing cerebellum: an investigation of the temporal pattern of alcohol and nicotine administration. Alcohol Clin. Exp. Res. 29, 437–442.
Collins, A.C., Wilkins, L.H., Slobe, B.S., Cao, J.Z., Bullock, A.E., 1996. Long-term ethanol and nicotine treatment elicit tolerance to ethanol. Alcohol Clin. Exp. Res. 20, 990–999.
Collins, A.C., Burch, J.B., de Fiebre, C.M., Marks, M.J., 1988. Tolerance to and cross tolerance between ethanol and nicotine. Pharmacol. Biochem. Behav. 29, 365–373.
Coronas, V., Durand, M., Chabot, J.G., Jourdan, F., Quirion, R., 2000. Acetylcholine induces neuritic outgrowth in rat primary olfactory bulb cultures. Neurosci. 98, 213–219.
Crabbe, J.C., Phillips, T.J., Buck, K.J., Cunningham, C.L., Belknap, J.K., 1999. Identifying genes for alcohol and drug sensitivity: recent progress and future directions. Trends Neurosci. 22, 173–179.
Crawley, J.N., Belknap, J.K., Collins, A., Crabbe, J.C., Frankel, W., Henderson, N., Hitzemann, R.J., Maxson, S.C., Miner, L.L., Silva, A.J., Wehner, J.M., Wynshaw-Boris, A., Paylor, R., 1997. Behavioral phenotypes of inbred mouse strains: implications and recommendations for molecular studies. Psychopharmacology (Berl) 132, 107–124.
Dani, J.A., Bertrand, D., 2007. Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. Annu. Rev. Pharmacol. Toxicol. 47, 699–729.
Dani, J.A., De Biasi, M., 2001. Cellular mechanisms of nicotine addiction. Pharmacol. Biochem. Behav. 70, 439–446.
Dani, J.A., Heinemann, S., 1996. Molecular and cellular aspects of nicotine abuse. Neuron. 16, 905–908.
Dawson, D.A., 2000. Drinking as a risk factor for sustained smoking. Drug Alcohol Depend 59, 235–249.
de Fiebre, C.M., Collins, A.C., 1993. A comparison of the development of tolerance to ethanol and cross-tolerance to nicotine following chronic ethanol treatment in Long-Sleep and Short-Sleep mice. J. Pharmacol. Exp. Ther. 266, 1398–1406.
de Fiebre, N.C., de Fiebre, C.M., 2005. Alpha7 Nicotinic acetylcholine receptor knockout selectively enhances ethanol-, but not beta-amyloid-induced neurotoxicity. Neurosci. Lett. 373, 42–47.
Degroot, A., Treit, D., 2002. Dorsal and ventral hippocampal cholinergic systems modulate anxiety in the plus-maze and shock-probe tests. Brain Res. 949, 60–70.
DiFranza, J.R., Guerrera, M.P., 1990. Alcoholism and smoking. J. Stud. Alcohol 51, 130–135.
Dohrman, D.P., Reiter, C.K., 2003. Ethanol modulates nicotine-induced upregulation of nAChRs. Brain Res 975, 90–98.
DiFranza, J., Savageau, J.A., Rigotti, N.A., Fletcher, K., Ockene, J.K., McNeill, A.D., Coleman, M., Wood, C., 2002. Development of symptoms of tobacco dependence in youths: 30-month follow-up data from the DANDY study. Tobacco Control 11, 228–235.
Doura, M.B., Gold, A.B., Keller, A.B., Perry, D.C., 2008. Adult and periadolescent rats differ in expression of nicotinic cholinergic receptor subtypes and in the response of these subtypes to chronic nicotine exposure. Brain Res., doi:10.1016/j.brainres.2008.03.056
Engin, E., Treit, D., 2007. The role of hippocampus in anxiety: intracerebral infusion studies. Behav. Pharmacol. 18, 365–374.
File, S.E., Kenny, P.J., Cheeta, S., 2000. The role of the dorsal hippocampal serotonergic and cholinergic systems in the modulation of anxiety. Pharmacol. Biochem. Behav. 66, 65–72.
Flores, C.M., Rogers, S.W., Pabreza, L.A., Wolfe, B.B., Kellar, K.J., 1992. A subtype of nicotinic cholinergic receptor in rat brain is composed of $\alpha4$ and $\beta2$ subunits and is upregulated by chronic nicotine treatment. Mol. Pharmacol. 41, 31–37.
Floyd, E.A., Young-Seigler, A.C., Ford, B.D., Reasor, J.D., Moore, E.L., Townsel, J.G., Rucker, H.K., 1997. Chronic ethanol ingestion produces cholinergic hypofunction in rat brain. Alcohol 14, 93–98.
Ford, B.D., Ivy, M.T., Mtshali, C.P., Townsel, J.G., 1999. The involvement of protein kinase C in the regulation of choline cotransport in Limulus. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 123, 255–261.
Forman, S.A., Righi, D.L., Miller, K.W., 1989. Ethanol increases agonist affinity for nicotinic receptors from Torpedo. Biochim. Biophys. Acta. 987, 95–103.
Gaddnas, H., Pietila, K., Ahtee, L., 2000. Effects of chronic oral nicotine treatment and its withdrawal on locomotor activity and brain monoamines in mice. Behav. Brain Res. 113, 65–72.
Gaimarri, A., Moretti, M., Riganti, L., Zanardi, A., Clementi, F., Gotti, C., 2007. Regulation of neuronal nicotinic receptor traffic and expression. Brain Res. Rev. 55, 134–143.
Gentry, C.L., Lukas, R.J., 2002. Regulation of nicotinic acetylcholine receptor numbers and function by chronic nicotine exposure. Curr. Drug Targets CNS Neurol. Disord 1, 359–385.
Gilbertson, R.J., Barron, S., 2005. Neonatal ethanol and nicotine exposure causes locomotor activity changes in preweanling animals. Pharmacol. Biochem. Behav 81, 54–64.
Gorbounova, O., Svensson, A.L., Jönsson, P., Mousavi, M., Miao, H., Hellström-Lindahl, E., Nordberg, A., 1998. Chronic ethanol treatment decreases [3H]epibatidine and [3H]nicotine binding and differentially regulates mRNA levels of nicotinic acetylcholine receptor subunits expressed in M10 and SH-SY5Y neuroblastoma cells. J. Neurochem. 70, 1134–1142.
Grant, K.A., 1994. Emerging neurochemical concepts in the actions of ethanol at ligand-gated ion channels. Behav. Pharmacol. 5, 383–404.
Grant, B.F., 1998. Age at smoking onset and its association with alcohol consumption and DSM-IV alcohol abuse and dependence: results from the national longitudinal alcohol epidemiologic survey. J. Substance Abuse 10, 59–73.
Happe, H.K., Murrin, L.C., 1992. High-affinity choline transport regulation by drug administration during postnatal development. J. Neurochem. 58, 2053–2059.
Happe, H.K., Peters, J.L., Bergman, D.A., Murrin, L.C., 1994. Localization of nicotinic cholinergic receptors in rat brain: autoradiographic studies with [3H]cytisine. Neurosci. 62, 929–944.
Hashimoto, E., Sakaguchi, S., Shiga, M., Ikeda, N., Toki, S., Saito, T., 2001. Epidemiological studies of tobacco smoking and dependence in Japan. Alcohol 24, 107–110.
Hasselmo, M.E., 2006. The role of acetylcholine in learning and memory. Curr. Opin. Neurobiol 16, 710–715.
Hohmann, C.F., Berger-Sweeney, J., 1998. Cholinergic regulation of cortical development and plasticity: new twists to an old story. Perspect. Dev. Neurobiol. 5, 401–425.
Hohmann, C.F., Brooks, A.R., Coyle, J.T., 1988. Neonatal lesions of the basal forebrain cholinergic neurons result in abnormal cortical development. Dev. Brain Res. 42, 253–264.
Howard, L.A., Ahluwalia, J.S., Lin, S.K., Sellers, E.M., Tyndale, R.F., 2003. CYP2E1*1D regulatory polymorphism: association with alcohol and nicotine dependence. Pharmacogenetics 13, 321–328.
Inoue, Y., Yao, L., Hopf, F.W., Fan, P., Jiang, Z., Bonci, A., Diamond, I., 2007. Nicotine and ethanol activate protein kinase A synergistically via G(i) betagamma subunits in nucleus

accumbens/ventral tegmental cocultures: the role of dopamine D(1)/D(2) and adenosine A(2A) receptors. J. Pharmacol. Exp. Ther. 322, 23–29.

Jang, M.H., Shin, M.C., Jung, S.B., Lee, T.H., Bahn, G.H., Kwon, Y.K., Kim, E.H., Kim, C.J., 2002a. Alcohol and nicotine reduce cell proliferation and enhance apoptosis in dentate gyrus. Neuro. Report 13, 1509–1513.

Jang, M.H., Shin, M.C., Lee, T.H., Kim, Y.P., Jung, S.B., Shin, D.H., Kim, H., Kim, S.S., Kim, E.H., Kim, C.J., 2002b. Alcohol and nicotine administration inhibits serotonin synthesis and tryptophan hydroxylase expression in dorsal and median raphe of young rats. Neurosci. Lett. 329, 141–144.

Johnston, L.D., O'Malley, P.M., Bachman, J.G., 2001. National Survey Results on Drug Use from the Monitoring the Future Study, 1975–2000. Department of Health and Human Services, Bethesda, MD, p. 1. NIH Publication No. 01-4924.

Klein, L.C., Stine, M.M., Pfaff, D.W., Vandenbergh, D.J., 2003. Maternal nicotine exposure increases nicotine preference in periadolescent male but not female C57B1/6J mice. Nicotine Tobacco Res. 5, 117–124.

Klein, L.C., Stine, M.M., Vandenbergh, D.J., Whetzel, C.A., Kamens, H.M., 2004. Sex differences in voluntary oral nicotine consumption by adolescent mice: a dose-response experiment. Pharmacol. Biochem. Behav. 78, 13–25.

Klemm, N., Kuhar, M.J., 1979. Post-mortem changes in high affinity choline uptake. J. Neurochem. 32, 1487–1494.

Kristofiková, Z., Platilová, V., Klaschka, J., 2003. Age- and sex-dependent effects of ethanol on hippocampal hemicholinium-3 sensitive choline carriers during postnatal development of rats. Neurochem. Res. 28, 397–405.

Larsson, A., Engel, J.A., 2004. Neurochemical and behavioral studies on ethanol and nicotine interactions. Neurosci. Biobehav. Rev. 27, 713–720.

Lau, C., Seidler, F.J., Cameron, A.M., Navarro, H.A., Bell, J.M., Bartolome, J., Slotkin, T.A., 1988. Nutritional influences on adrenal chromaffin cell development: comparison with central neurons. Pediatr. Res. 24, 583–587.

Lê, A.D., Li, Z., Funk, D., Shram, M., Li, T.K., Shaham, Y., 2006. Increased vulnerability to nicotine self-administration and relapse in alcohol-naive offspring of rats selectively bred for high alcohol intake. J. Neurosci. 26, 1872–1879.

Levin, E.D., Slotkin, T.A., 1998. Developmental neurotoxicity of nicotine. In: Slikker, W., Chang, L.W. (Eds.), Handbook of Developmental Neurotoxicology. Academic Press, San Diego, pp. 587–615.

Lopez, M., Simpson, D., White, N., Randall, C., 2003. Age- and sex-related differences in alcohol and nicotine effects in C57BL/6J mice. Addict. Biol. 8, 419–427.

Mansvelder, H.D., McGehee, D.S., 2002. Cellular and synaptic mechanisms of nicotine addiction. J. Neurobiol. 53, 606–617.

Marks, J.L., Hill, E.M., Pomerleau, C.S., Mudd, S.A., Blow, F.C., 1997. Nicotine dependence and withdrawal in alcoholic and nonalcoholic ever-smokers. J. Subst. Abuse Treat. 14, 521–527.

Marttila, K., Raattamaa, H., Ahtee, L., 2006. Effects of chronic nicotine administration and its withdrawal on striatal FosB/DeltaFosB and c-Fos expression in rats and mice. Neuropharmacol. 51, 44–51.

Meyerhoff, D.J., Tizabi, Y., Staley, J.K., Durazzo, T.C., Glass, J.M., Nixon, S.J., 2006. Smoking comorbidity in alcoholism: neurobiological and neurocognitive consequences. Alcohol. Clin. Exp. Res. 30, 253–264.

Miller, N.S., Gold, M.S., 1998. Comorbid cigarette and alcohol addiction: epidemiology and treatment. J. Addict Dis 17, 55–66.

Nashmi, R., Xiao, C., Deshpande, P., McKinney, S., Grady, S.R., Whiteaker, P., Huang, Q., McClure-Begley, T., Lindstrom, J.M., Labarca, C., Collins, A.C., Marks, M.J., Lester, H.A., 2007. Chronic nicotine cell specifically upregulates functional a4* nicotinic receptors: basis for both tolerance in midbrain and enhance long-term potentiation in perforant path. J. Neurosci. 27, 8202–8218.

National Institute on Drug Abuse, 1998. Nicotine Addiction, p. 7. NIH Publication Number 98-4342, Washington, DC.

Navarro, H.A., Seidler, F.J., Eylers, J.P., Baker, F.E., Dobbins, S.S., Lappi, S.E., Slotkin, T.A., 1989. Effects of prenatal nicotine exposure on development of central and peripheral cholinergic neurotransmitter systems. Evidence for cholinergic trophic influences in developing brain. J. Pharmacol. Exp. Ther 251, 894–900.

Nelson, D.E., Giovino, G.A., Shopland, D.R., Mowery, P.D., Mills, S.L., Eriksen, M.P., 1995. Trends in cigarette smoking among US adolescents, 1974 through 1991. Am. J. Public Health 85, 34–40.

Nestler, E.J., 2001. Psychogenomics: opportunities for understanding addiction. J. Neurosci. 21, 8324–8327.

Nuutinen, S., Ahtee, L., Tuominen, R.K., 2005. Time and brain region specific up-regulation of low affinity neuronal nicotinic receptors during chronic nicotine administration in mice. Eur. J. Pharmacol. 515, 83–89.

Oneta, C.M., Simanowski, U.A., Martinez, M., Allali-Hassani, A., Parés, X., Homann, N., Conradt, C., Waldherr, R., Fiehn, W., Coutelle, C., Seitz, H.K., 1998. First pass metabolism of ethanol is strikingly influenced by the speed of gastric emptying. Gut. 43, 612–619.

Parnell, S.E., West, J.R., Chen, W.J., 2006. Nicotine decreases blood alcohol concentrations in adult rats: a phenomenon potentially related to gastric function. Alcohol. Clin. Exp. Res. 30, 1408–1413.

Patten, C.A., Martin, J.E., Owen, N., 1996. Can psychiatric and chemical dependency treatment units be smoke free? J. Subst. Abuse Treat. 13, 107–118.

Penland, S., Hoplight, B., Obernier, J., Crews, F.T., 2001. Effects of nicotine on ethanol dependence and brain damage. Alcohol 24, 45–54.

Picciotto, M.R., Addy, N.A., Mineur, Y.S., Brunzell, D.H., 2008. It is not "either/or": activation and desensitization of nicotinic acetylcholine receptors both contribute to behaviors related to nicotine addiction and mood. Prog. Neurobiol. 84, 329–342.

Pugh, P.C., Margiotta, J.F., 2000. Nicotinic acetylcholine receptor agonists promote survival and reduce apoptosis of chick ciliary ganglion neurons. Mol. Cell. Neurosci. 15, 113–122.

Rakic, P., Bourgeois, J.P., Goldman-Rakic, P.S., 1994. Synaptic development of the cerebral cortex: implications for learning, memory, and mental illness. Prog. Brain Res. 102, 227–243.

Rhodes, M.C., Seidler, F.J., Qiao, D., Tate, C.A., Cousins, M.M., Thillai, I., Slotkin, T.A., 2003. Does pharmacotherapy for preterm labor sensitize the developing brain to environmental neurotoxicants? Cellular and synaptic effects of sequential exposure to terbutaline and chlorpyrifos in neonatal rats. Toxicol. Appl. Pharmacol. 195, 203–217.

Ricceri, L., Hohmann, C., Berger-Sweeney, J., 2002. Early neonatal 192 IgG saporin induces learning impairments and disrupts cortical morphogenesis in rats. Brain Res. 954, 160–172.

Robles, N., Sabriá, J., 2008. Effects of moderate chronic ethanol consumption on hippocampal nicotinic receptors and associative learning. Neurobiol. Learn. Mem. 89, 497–503.

Saltarelli, M.D., Yamada, K., Coyle, J.T., 1990. Phospholipase A2 and (3H)hemicholinium-3 binding sites in rat brain: a potential second-messenger role for fatty acids in the regulation of high-affinity choline uptake. J. Neurosci. 10, 62–72.

Schorling, J.B., Gutgesell, M., Klas, P., Smith, D., Keller, A., 1994. Tobacco, alcohol and other drug use among college students. J. Subst. Abuse 6, 105–115.

Scott, A.M., Kellow, J.E., Shuter, B., Nolan, J.M., Hoschl, R., Jones, M.P., 1993. Effects of cigarette smoking on solid and liquid intragastric distribution and gastric emptying. Gastroenterology 104, 410–416.

Silveri, M.M., Spear, L.P., 2000. Ontogeny of ethanol elimination and ethanol-induced hypothermia. Alcohol. 20, 45–53.

Simon, J.R., Atweh, S., Kuhar, M.J., 1976. Sodium-dependent high affinity choline uptake: a regulatory step in the synthesis of acetylcholine. J. Neurochem. 26, 909–922.

Sircar, R., Sircar, D., 2006. Repeated ethanol treatment in adolescent rats alters cortical NMDA receptor. Alcohol 39, 51–58.
Slotkin, T.A., 2002. Nicotine and the adolescent brain: insights from an animal model. Neurotoxicol. Teratol. 24, 369–384.
Slotkin, T.A., 2004. Cholinergic systems in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates. Toxicol. Appl. Pharmachol. 198, 132–151.
Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.
Snedecor, G.W., Cochran, W.G., 1967. Statistical Methods, 6th ed. Iowa State University Press, Ames, Iowa.
Soderpalm, B., Ericson, M., Olausson, P., Blomqvist, O., Engel, J.A., 2000. Nicotinic mechanisms involved in the dopamine activating and reinforcing properties of ethanol. Behav. Brain Res. 113, 85–96.
Song, M., Wang, X.Y., Zhao, M., Wang, X.Y., Zhai, H.F., Lu, L., 2008. Role of stress in acquisition of alcohol-conditioned place preference in adolescent and adult mice. Alcohol Clin. Exp. Res. 31, 2001–2005 Erratum in: Alcohol Clin. Exp. Res. 2008. 32, 184.
Sparks, J.A., Pauly, J.R., 1999. Effects of continuous oral nicotine administration on brain nicotinic receptors and responsiveness to nicotine in C57B1/6 mice. Psychopharmacology 141, 145–153.
Spear, L., 2000a. Modeling adolescent development and alcohol use in animals. Alcohol Res. Health 24, 115–123.
Spear, L.P., 2000b. The adolescent brain and age-related behavioral manifestations. Neurosci. Biobehav. Rev 24, 417–463.
Sun, G.Y., Sun, A.Y., 1985. Ethanol and membrane lipids. Alcohol. Clin. Exp. Res 9, 164–180.
Swan, G.E., Carmelli, D., Rosenman, R.H., Fabisz, R., 1990. Smoking and alcohol consumption in adult male twins: genetic heritability and shared environmental influences. J. Subst. Abuse 2, 39–50.
Tammimaki, A., Pietila, K., Raattamaa, H., Ahtee, L., 2006. Effect of quinpirole on striatal dopamine release and locomotor activity in nicotine-treated mice. Eur. J. Pharmacol. 531, 118–125.
Tizabi, Y., Al-Namaeh, M., Manaye, K.F., Taylor, R.E., 2003. Protective effects of nicotine on ethanol-induced toxicity in cultured cerebellar granule cells. Neurotox. Res. 5, 315–321.
Tizabi, Y., Manaye, K.F., Smoot, D.T., Taylor, R.E., 2004. Nicotine inhibits ethanol-induced tocixity in cultured cerebral cortical cells. Neurotox. Res. 6, 311–316.
Tizabi, Y., Manaye, K.F., Taylor, R.E., 2005. Nicotine blocks ethanol-induced apoptosis in primary cultures of rat cerebral cortical and cerebellar granule cells. Neurotox. Res. 7, 319–322.
Tizabi, Y., Bai, L., Copeland, R.L.J., Taylor, R.E., 2007. Combined effects of systemic alcohol and nicotine on dopamine release in the nucleus accumbens shell. Alcohol Alcohol 42, 413–416.
Torabi, M.R., Bailey, W.J., Majd-Jabbari, M., 1993. Cigarette smoking as a predictor of alcohol and other drug use by children and adolescents: evidence of the 'Gateway Drug Effect'. J. Sch. Health 63, 302–306.
Trauth, J.A., Seidler, F.J., McCook, E.C., Slotkin, T.A., 1999. Adolescent nicotine exposure causes persistent upregulation of nicotinic cholinergic receptors in rat brain regions. Brain Res. 851, 9–19.
Trauth, J.A., McCook, E.C., Seidler, F.J., Slotkin, T.A., 2000a. Modeling adolescent nicotine exposure: effects on cholinergic systems in rat brain regions. Brain Res. 873, 18–25.
Trauth, J.A., Seidler, F.J., Slotkin, T.A., 2000b. An animal model of adolescent nicotine exposure: effects on gene expression and macromolecular constituents in rat brain regions. Brain Res. 867, 29–39.
Trauth, J.A., Seidler, F.J., Slotkin, T.A., 2000c. Persistent and delayed behavioral changes after nicotine treatment in adolescent rats. Brain Res. 880, 167–172.
Vihavainen, T., Mijatovic, J., Piepponen, T.P., Tuominen, R.K., Ahtee, L., 2006. Effect of morphine on locomotor activity and striatal monoamine metabolism in nicotine-withdrawn mice. Behav. Brain Res. 173, 85–93.
Viveros, M.P., Marco, E.M., Llorente, R., Lamota, L., 2007. The role of the hippocampus in mediating emotional responses to nicotine and cannabinoids: a possible neural substrate for functional interactions. Behav. Pharmacol. 18, 375–389.
Whiting, P., Lindstrom, J., 1987. Purification and characterization of a nicotinic acetylcholine receptor from rat brain. Proc. Natl. Acad. Sci. 84, 595–599.
Wonnacott, S., 1997. Presynaptic nicotinic ACh receptors. Trends Neurosci. 20, 92–98.
Wood, T., Wewers, M.E., Groner, J., Ahijevych, K., 2004. Smoke constituent exposure and smoking topography of adolescent daily cigarette smokers. Nicotine Tob. Res. 6, 853–862.
Wu, G., Tonner, P.H., Miller, K.W., 1993. Ethanol stabilizes the open channel stat of the Torpedo nicotinic acetylcholine receptor. Mol. Pharmacol. 45, 102–108.
Xu, Z., Seidler, F.J., Tate, C.A., Garcia, S.J., Slikker, W., Slotkin, T.A., 2003. Sex-selective hippocampal alterations after adolescent nicotine administration: effects on neurospecific proteins. Nicotine Tobacco Res. 5, 955–960.
Yu, D., Zhang, L., Eiselé, J.L., Bertrand, D., Changeux, J.P., Weight, F.F., 1996. Ethanol inhibition of nicotinic acetylcholine type alpha 7 receptors involves the amino-terminal domain of the receptor. Mol. Pharmacol. 50, 1010–1016.
Zahalka, E., Seidler, F.J., Lappi, S.E., Yanai, J., Slotkin, T.A., 1993. Differential development of cholinergic nerve terminal markers in rat brain regions: implications for nerve terminal density, impulse activity and specific gene expression. Brain Res. 601, 221–229.
Zahalka, E.A., Seidler, F.J., Lappi, S.E., McCook, E.C., Yanai, J., Slotkin, T.A., 1992. Deficits in development of central cholinergic pathways caused by fetal nicotine exposure: differential effects on choline acetyltransferase activity and [^{3}H]hemicholinium-3 binding. Neurotoxicol. Teratol. 14, 375–382.

5. ARTIGO 2 – Exposição combinada de nicotina e etanol em camundongos adolescentes: efeitos no sistema colinérgico após curto e longo período de retirada.

O segundo artigo apresentado nesta tese foi aceito para publicação este ano no periódico internacional *Neuroscience* (fator de impacto 2008=3,556), sendo intitulado: "Combined exposure to nicotine and ethanol in adolescent mice: Effects on the central cholinergic systems during short and long term withdrawal" (doi:10.1016/j.neuroscience.2009.05.032). Além disso, os resultados deste trabalho serão apresentados no congresso nacional da Federação de Sociedades de Biologia Experimental (XXIV Reunião Anual da FeSBE).

Considerando que o conhecimento dos substratos biológicos afetados durante o período de retirada é fundamental para prevenir as recaídas pela droga, neste trabalho tivemos como objetivo avaliar o sistema colinérgico de camundongos adolescentes após a retirada da exposição de nicotina e/ou etanol. Em resumo, após curto período de retirada (PN50) os grupos NIC e NIC+ETOH apresentaram *upregulation* dos nAChRs. Ainda em PN50, animais previamente expostos à nicotina apresentaram aumento da atividade da ChAT, que foi revertido no grupo NIC+ETOH. Em relação ao transportador pré-sinaptico de colina, houve redução dos seus níveis mesmo após um mês de retirada de ETOH e NIC+ETOH. Desta forma, este estudo fornece evidências experimentais das interações entre nicotina e etanol existentes no sistema colinérgico central durante o período de retirada.

Neuroscience 162 (2009) 1174–1186

COMBINED EXPOSURE TO NICOTINE AND ETHANOL IN ADOLESCENT MICE: EFFECTS ON THE CENTRAL CHOLINERGIC SYSTEMS DURING SHORT AND LONG TERM WITHDRAWAL

A. RIBEIRO-CARVALHO, C. S. LIMA, A. H. MEDEIROS, N. R. SIQUEIRA, C. C. FILGUEIRAS, A. C. MANHÃES AND Y. ABREU-VILLAÇA*

Laboratório de Neurofisiologia, Departamento de Ciências Fisiológicas, Instituto de Biologia Roberto Alcantara Gomes, Universidade do Estado do Rio de Janeiro, Av. Prof. Manoel de Abreu 444, 5 andar, Vila Isabel, Rio de Janeiro, RJ, 20550-170, Brazil

Abstract—Relapse to drug use is a major public health problem. In this sense, understanding the biological substrates that are affected during withdrawal may provide information to prevent relapse. Both smoking and alcoholic beverage consumption usually begin during adolescence, however, little is known about the basic neurobiology of the combined adolescent exposure, particularly during withdrawal. Since nicotine is a cholinergic agonist and it has been shown that ethanol interferes with nicotinic acetylcholine receptors (nAChRs), the current study focused on the effects of drug withdrawal on the central cholinergic system. From the 30th to the 45th postnatal day (PN), C57BL/6 male and female mice were exposed to nicotine free base (NIC) and/or ethanol (ETOH). Four groups were analyzed: (1) concomitant NIC (50 μg/ml in 2% saccharin to drink) and ETOH (25%, 2 g/kg i.p. injected every other day) exposure; (2) NIC exposure; (3) ETOH exposure; (4) vehicle. We assessed nAChR binding, choline acetyltransferase (ChAT) activity and [^{3}H]hemicholinium-3 (HC-3) binding in the cerebral cortex and midbrain of mice at short (PN50) and long term (PN75) withdrawal. NIC and NIC+ETOH promoted nAChR upregulation during a short-term withdrawal. NIC short-term withdrawal elicited an increase in ChAT activity that was reversed by ETOH withdrawal. In addition, NIC+ETOH elicited a decrease in ChAT activity at long term withdrawal. Regarding HC-3, ETOH and NIC+ETOH promoted a decrease that persisted at long-term withdrawal. The present study provides experimental evidence that nicotine and ethanol during adolescence interact resulting in cholinergic system alterations during withdrawal.
© 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: adolescence, nicotinic acetylcholine receptors, alcohol, tobacco, development, abstinence.

Epidemiological studies have indicated that nicotine and ethanol are the most commonly used and abused drugs and that a close relationship between smoking and alcohol consumption exists (DiFranza and Guerrera, 1990; Larsson and Engel, 2004). Tobacco smoking and alcohol consumption typically begin during adolescence (National Institute on Drug Abuse, 1998; Nelson et al., 1995; Spear, 2000a) and it is now clear that adolescents show a peculiar sensitivity to both nicotine and ethanol (Spear, 2000a; Slotkin, 2002). Studies have confirmed that adolescent drinkers are more likely than nondrinkers to be smokers and vice versa (Torabi et al., 1993; Schorling et al., 1994; Chen and Kandel, 1995) and have also shown that there is a strong correlation between onset of tobacco consumption at an early age and alcohol addiction (DiFranza and Guerrera, 1990; Grant, 1998).

Cessation is the only effective measure to prevent or limit the long-term negative effects of smoking and drinking (WHO, 2004; De Biasi and Salas, 2008). Most smokers and alcoholic beverage abusers recognize the negative impact of drug abuse on health and would prefer to quit, if possible. However, in spite of many attempts, very few actually succeed (Gulliver et al., 2006; De Biasi and Salas, 2008). A major obstacle to long term abstinence is the presence of withdrawal symptoms. In fact, the duration and severity of withdrawal are strong predictors of relapse to drug use (West et al., 1989; Piasecki et al., 2003). The relationship between nicotine and ethanol addiction can also be demonstrated during withdrawal. Smoking interruption is more difficult to accomplish in former or current alcohol abusers (Bobo et al., 1987) and smoking cessation has been associated with altered ethanol consumption (Carmelli et al., 1993; Friend and Pagano, 2005). Altogether, these findings indicate that a better understanding of the neurobiological substrates affected by nicotine and/or ethanol exposure and withdrawal during adolescence may help in the development of new pharmacological therapies to prevent relapse and/or reverse the addictive state.

Among tobacco components, nicotine has been shown to be responsible for a wide range of nervous system effects, including tobacco addiction (Benowitz, 1992; Dani and Heinemann, 1996). As an acetylcholine analog, the ionotropic nicotinic acetylcholine receptors (nAChRs) are the primary cellular mediators of nicotine's effects. As for ethanol, it directly influences the function of various ligand-gated ion channels, including nAChRs (for review: Grant, 1994), which suggests that the nAChR is a site at which nicotine and ethanol interact. In fact, we have previously demonstrated in mice that concomitant nicotine and ethanol exposure during adolescence elicits stronger upregulation of $\alpha 4\beta 2$-nAChRs in the midbrain and cerebral cortex

*Corresponding author. Tel: +5521-2587-6295; fax: +5521-2587-6129.
E-mail address: yael_a_v@yahoo.com.br (Y. Abreu-Villaça).

Abbreviations: ANOVA, analysis of variance; BEC, blood ethanol concentration; ChAT, choline acetyltransferase; ETOH, ethanol-exposed group; FPLSD, Fisher's protected least significant difference; HC-3, [^{3}H]hemicholinium-3; nAChR, nicotinic acetylcholine receptor; NIC, nicotine-exposed group; NIC+ETOH, nicotine and ethanol-exposed group; PN, postnatal day; rANOVA, repeated-measures analysis of variance; VEH, vehicle group.

0306-4522/09 $ - see front matter © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2009.05.032

when compared to alterations elicited by either drug alone (Ribeiro-Carvalho et al., 2008), thus providing experimental evidence that nicotine and ethanol interactions are mediated, at least in part, by the central cholinergic system.

Interestingly, it has been proposed that the $\beta2$*-nAChRs, but not $\alpha5$*- or $\alpha7$*-nAChRs, may contribute to affective components of the nicotine withdrawal syndrome in mice (Jackson et al., 2008). In the same way, Butt et al. (2004) demonstrated that $\alpha4$*-nAChRs modulate ethanol withdrawal in mice. Despite these findings, there have been relatively few animal studies on the basic neurobiology of nicotine and ethanol withdrawal, particularly in animal models of adolescent exposure. In this regard, we have previously examined the effects of adolescent nicotine and/or ethanol administration on memory/learning (Abreu-Villaça et al., 2007) and anxiety (Abreu-Villaça et al., 2008) during drug administration and withdrawal. These studies provided experimental evidence for functional interactions between nicotine and ethanol in the regulation of behavioral responses during withdrawal.

The purpose of the current study was to examine the effects of adolescent nicotine and/or ethanol withdrawal on the central cholinergic neurotransmitter system. It complements our previous findings regarding the effects of nicotine and/ethanol during exposure (Ribeiro-Carvalho et al., 2008). In keeping with earlier experimental designs (Abreu-Villaça et al., 2006, 2007, 2008; Ribeiro-Carvalho et al., 2008), we chose to give animals free access to a nicotine solution in the drinking water, which allows for consumption during their active time. For ethanol, we chose a moderate dose to be injected (i.p.) every other day, mimicking adolescents' binge drinking. Exposure began on postnatal day (PN) 30, which, based on brain development, onset of puberty, and patterns of drug reactivity, represents the early adolescent stage in the rodent, and extended up to PN45, still well within adolescence (Spear, 2000b). We evaluated three cholinergic biomarkers at short-term (five days post-exposure) and long term (1 month post-exposure) withdrawal: First, we assessed nAChR binding with [^{3}H]cytisine, a ligand that binds selectively to the $\alpha4\beta2$-nAChR, the predominant subtype in mammalian brain (Flores et al., 1992). We also assessed choline acetyltransferase (ChAT) activity and the binding of [^{3}H]hemicholinium-3 (HC-3) to the high-affinity presynaptic choline transporter. ChAT, the enzyme responsible for acetylcholine biosynthesis, is a constitutive marker for cholinergic nerve terminals and serves as an archetypal measure of cholinergic innervation, but its activity does not respond to changes in impulse flow. Accordingly, ChAT reflects the concentration of cholinergic nerve terminals (Navarro et al., 1989; Happe and Murrin, 1992b; Zahalka et al., 1992, 1993; Aubert et al., 1996). In contrast, high affinity choline uptake, as assessed with the binding of HC-3 to the presynaptic high-affinity choline transporter, is responsive to neuronal activity (Simon et al., 1976; Klemm and Kuhar, 1979) and the comparative changes in ChAT and HC-3 binding allows for the distinction between effects on the concentration of synaptic terminals and effects on synaptic activity (Navarro et al., 1989; Happe and Murrin, 1992b; Zahalka et al., 1992, 1993; Aubert et al., 1996).

EXPERIMENTAL PROCEDURES

Animal treatment

All experiments were carried out in accordance with the declaration of Helsinki and with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health. All procedures were approved by the Institute of Biology/UERJ Ethical Committee for Animal Research, minimizing the number of animals used and avoiding animal suffering. C57BL/6 mice were chosen because prior reports, which used this strain and the same concentration of nicotine as the one used in the present study, demonstrated that this dose of nicotine is well tolerated by this strain since it does not elicit marked reductions in fluid intake (Sparks and Pauly, 1999; Klein et al., 2003, 2004; Abreu-Villaça et al., 2007, 2008; Manhães et al., 2008; Ribeiro-Carvalho et al., 2008). All mice were bred and maintained in our laboratory. Animals were derived from a C57BL/6 colony maintained at the Universidade Estadual de Campinas (São Paulo, Brazil) for over 70 generations. The animals were kept in a temperature-controlled room on a 12-h light/dark cycle (lights on at 2:00 AM). Access to food and water was *ad libitum*. On the first postnatal day (PN1), litters were culled to a maximum of eight mice to ensure standard nutrition. At weaning (PN25) animals were separated by sex and allowed free access to food and water.

On PN29, pups from 28 litters (50 females and 61 males) were individually housed, which allowed for accurate individual measurement of fluid intake/nicotine consumption. Animals were exposed to nicotine and/or ethanol from PN30 to PN45, the approximate age range during which animals of both genders and most breeding stock exhibit adolescent-typical behavioral characteristics and particular neurochemical and endocrine patterns when compared to adulthood and pre-pubertal periods (Spear, 2000b). During this period, 25% ethanol (2 g/kg) solution (v/v) in saline or saline only was injected (i.p.) every other day in order to mimic cyclical patterns of alcohol consumption; while (−)-nicotine free base (50 μg/ml) in 2% saccharin or 2% saccharin only was administered in the drinking water (the sole source of fluid) in order to mimic intermittent nicotine consumption. Accordingly, each one of the following treatment groups was composed of no more than one male and one female mouse from each litter: vehicle group (VEH) (oral saccharin+injected saline), ethanol-exposed group (ETOH) (oral saccharin+injected ethanol), nicotine-exposed group (NIC) (oral nicotine/saccharin+injected saline) and those receiving the combined treatment: nicotine and ethanol-exposed group (NIC+ETOH) (oral nicotine/saccharin+injected ethanol). Bottles were cleaned and refilled daily. Loss due to leakage was measured from a bottle placed in an empty cage ("blank"), and subtracted from fluid consumption data. Body weights and fluid consumption were also measured every day. Since body weight increases significantly during adolescence, daily fluid intake data were obtained by dividing the absolute values of fluid intake of each animal by its own body weight.

Blood ethanol concentration (BEC) and cotinine plasma levels

BEC and cotinine plasma levels were assessed in two groups specifically treated for these analyses. Animals in these groups were not used for the neurochemical analyses. One group of mice was treated with nicotine and/or ethanol as described above. Thirty and 120 minutes after the last injection of ethanol (at PN44), animals were decapitated and the blood collected from VEH (30 min: n=7 and 120 min: n=7), ETOH (30 min: n=10 and 120 min: n=10) and NIC+ETOH (30 min: n=9 and 120 min: n=9) exposed mice. Blood was centrifuged at 2000×g for 20 min and supernatant stored at 4 °C

until assayed. BEC was assessed using an enzymatic kit (Alcohol Reagent Set, Pointe Scientific Inc., Canton, MI, USA) in accordance with the manufacturer's recommendations.

A second separate group of mice was treated with nicotine and/or ethanol as described above. At the end of treatment (PN45), 1 h after the beginning of the dark cycle, animals were decapitated and the blood collected in heparinized syringes from VEH (n=4), NIC (n=9), ETOH (n=4) and NIC+ETOH (n=9) exposed mice. Blood was centrifuged for 10 min at 2000×g at 4 °C and plasma was stored at −20 °C. Cotinine plasma levels were determined using a cotinine assay kit from Orasure Technologies (Bethlehem, PA, USA) in accordance with the manufacturer's recommendations. These techniques were used to evaluate whether there are pharmacokinetic interactions between nicotine and ethanol, which, if present, could possibly influence the biochemical results.

Tissue preparation and assays

Studies were conducted after the end of the drug administration period at two time points: during a short-term withdrawal (five days post-exposure, PN50) and during a long-term withdrawal (1 month post-exposure, PN75). Animals were decapitated and the brain regions were dissected by making blunt cuts through the cerebellar peduncles, whereupon the cerebellum (including flocculi) was lifted from the underlying tissue. The cerebral cortex was separated from the midbrain+brain stem by a cut made caudal to the thalamus. The midbrain was then dissected from the hind-brain by making a cut caudal to the inferior colliculus, so that the midbrain contained the entire dorsal raphe nucleus but not descending serotonergic nuclei (Fumagalli et al., 1996; Trauth et al., 2000b). After tissue dissection, cerebral cortex and midbrain were frozen in liquid nitrogen, and stored at −45 °C until assayed. For each treatment group and age, 12–15 animals were examined, equally divided into males and females.

Tissues were thawed and homogenized (Ultra-Turrax T10 basic, IKA, São Paulo, SP, Brazil) in 40 volumes of ice cold 50 mM Tris HCl (pH 7.4) and aliquots were withdrawn for measurements of ChAT activity (Lau et al., 1988) and total protein (Smith et al., 1985). To prepare the cell membrane fraction, the homogenates were sedimented at 40,000×g for 10 min and the supernatant solution was discarded. The membrane pellet was resuspended (Ultra-Turrax) in the original volume of buffer, resedimented, and the resultant pellet was resuspended in approximately 10 volumes (based on the original weight of the tissue) of the same buffer using a smooth glass homogenizer fitted with a Teflon pestle. Aliquots of this resuspension were withdrawn for measurements of [^{3}H]cytisine and [^{3}H]HC-3 binding, and for membrane protein.

All assays have been described in detail in previous papers (Trauth et al., 1999, 2000a; Abreu-Villaça et al., 2003a, 2004b) and will therefore be presented briefly. ChAT activity was assayed in tissue homogenate using 50 μM [^{14}C]acetyl-coenzyme A. Labeled acetylcholine was then extracted and the activity determined relative to tissue protein. [^{3}H]Cytisine and [^{3}H]HC-3 binding were determined in the cell membrane fraction using final ligand concentrations of 2 nM; specific binding was displaced with 100 μM nicotine for nAChRs and 20 μM unlabeled HC-3 for the high affinity choline transporter. Values were then calculated as specific binding per mg of membrane protein.

We used a single ligand concentration that lies above the Kd but nevertheless below full saturation of the binding site for determinations of nicotinic receptor and presynaptic choline transporter binding. The strategy of using a single, subsaturating ligand concentration enables the detection of drug-induced changes regardless of whether the changes are in affinity (Kd) or capacity ($B_{max.}$), but does not distinguish between the two mechanisms. The interpretation of results of the present study does not depend upon whether the change is specific to concentration or affinity. However, previous studies demonstrated that nicotine and ethanol affect mainly the concentration of nAChRs and HC-3 binding sites (Trauth et al., 1999; Dohrman and Reiter, 2003).

Materials

Radioisotopically-labeled compounds came from PerkinElmer Life Sciences (Boston, MA, USA): [^{14}C]acetyl-CoA (specific activity 4 mCi/mmol), [^{3}H]HC-3 (specific activity 144.5 Ci/mmol) and [^{3}H]cytisine (specific activity, 40 Ci/mmol). Sigma Chemical Co. (St. Louis, MO, USA) was the source for bovine albumin, BCA kit, eserine hemisulfate salt, 3-heptanone, (−)-nicotine hydrogen tartate salt, (−)-nicotine free base, sodium tetraphenylborate and Triton X-100. VETEC Química Fina Ltda. (Rio de Janeiro, RJ, Brazil) was the source for all other reagents.

Data analysis

Fluid intake/body weight, body weight, brain region weight, BEC and cotinine levels. Initially, repeated-measures analyses of variance (rANOVA) on each variable (fluid intake/body weight and body weight) were carried out. *Treatment* and *Sex* were used as between-subjects factors. *Day* was considered the within-subjects factor. Whenever significant *Treatment* effects or interactions were detected, lower order analyses of variance (ANOVAs) followed by pairwise post hoc analyses (Fisher's protected least significant difference—FPLSD) were carried out. Effects of *Treatment* on brain region weights, BEC and cotinine levels were analyzed with separate ANOVAs.

Cholinergic biomarkers. To reduce the likelihood of type 1 statistical errors that might result from repeated testing of the global data set, we first performed a rANOVA (data log-transformed whenever variance was heterogeneous) on all factors: *Treatment, Brain Region, Age* and *Sex*. The *Cholinergic Biomarkers* (nAChR binding, ChAT, HC-3 binding) were treated as repeated measures since each was obtained from the same tissue homogenate. Whenever this initial test indicated treatment effects that differed among the different biomarkers, ages, brain regions and sexes, data were then re-examined separately using ANOVAs followed by pairwise post hoc analyses (FPLSD); however, where treatment effects did not interact with other variables only the main effect was recorded without testing of individual differences. The same procedures were performed for the analysis of the development of the cholinergic system, except for the fact that the *Treatment* factor was not included, since only VEH mice data were used to run the ANOVAs.

Nicotine and ethanol interactions. The one-dimensional statistical design described above, in which *Treatment* (VEH, ETOH, NIC and NIC+ETOH) was considered the between-subjects factor, was used to verify whether significant differences between the four treatment groups existed. However, the combined use of nicotine and ethanol can have more-than-additive (synergistic), less-than-additive or additive effects which are not addressed by the one-dimensional design. Therefore, a two-dimensional design (Rhodes et al., 2003; Abreu-Villaça et al., 2004a,b, 2007, 2008; Ribeiro-Carvalho et al., 2008) was used. In this design, *Ethanol* (treated: ETOH and NIC+ETOH; non-treated: VEH and NIC) was considered one of the between-subjects factors. *Nicotine* (treated: NIC and NIC+ETOH; non-treated: VEH and ETOH) was considered the other between-subjects factors. In this formulation, more-than-additive (synergistic) and less-than-additive effects appear as significant interactions between the two treatment dimensions, whereas simple, additive effects do not show significant interactions.

Significance was assumed at the level of $P<0.05$ for main effects; however, for interactions at $P<0.1$, we also examined whether lower-order main effects were detectable after subdivision of the interactive variables (Snedecor and Cochran, 1967).

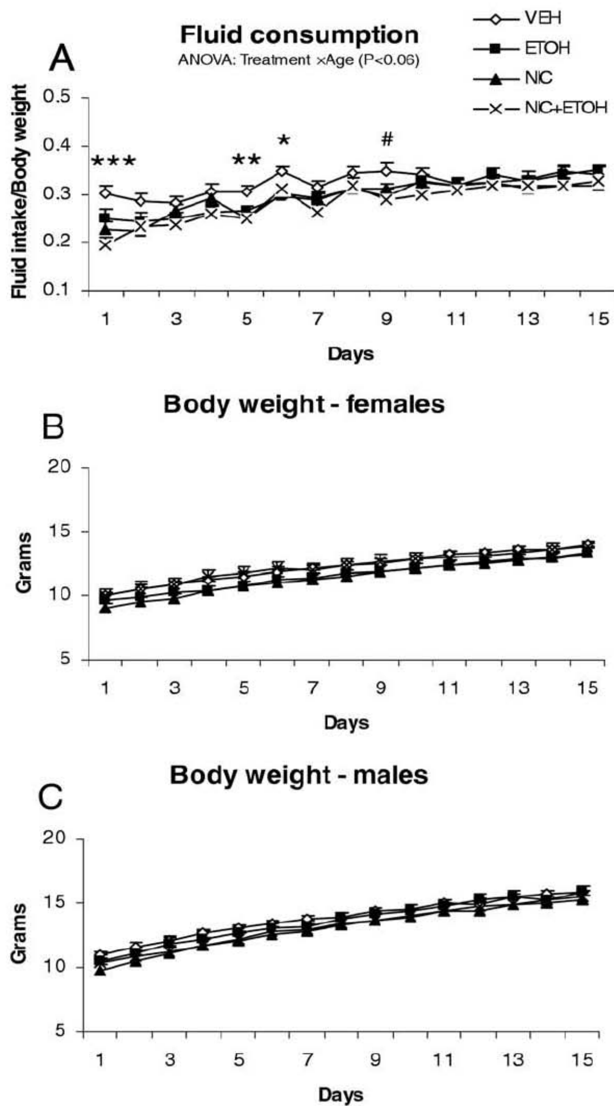

Fig. 1. Effects of adolescent nicotine and/or, ethanol on fluid consumption (A) and body weight (B, C) during adolescent exposure (PN30-45). Data are presented as the percent change from control values collapsed across regions. Significant differences between groups as revealed by FPLSD. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ VEH vs. NIC, ETOH and NIC+ETOH groups. # $P<0.01$ VEH vs. ETOH and NIC+ETOH groups. Values are means±SEM.

Data are compiled as means and standard errors. To facilitate comparisons, in addition to the presentation of the raw data, the effects of each treatment are also given as the percentage change from the corresponding control group. Statistical comparisons were made on raw data.

RESULTS

Development of the cholinergic system

Data from VEH mice were submitted to a global rANOVA across all cholinergic biomarkers, brain regions, ages and sexes. A *Cholinergic Biomarker×Region×Age* interaction ($F=9.0$, $df=2$, $P<0.001$). Given this result, data analysis was carried out separately on each biochemical marker and then the results were reexamined. The factors *Brain Region, Age and Sex* were kept in the analysis.

In VEH mice, $\alpha 4\beta 2$-nAChR binding was dependent on the region and age (*Region*: $F=265.9$, $df=1$, $P<0.001$; *Region×Age*: $F=48.6$, $df=1$, $P<0.03$). Separate analyses for each region indicated that, in the cerebral cortex, binding was higher in PN50 when compared to PN75 mice (*Age*: $F=20.5$, $df=1$, $P<0.001$), while in the midbrain, higher values were present at PN75 (*Age*: $F=31.5$, $df=1$, $P<0.001$).

The analysis of ChAT activity in VEH mice demonstrated *Region* ($F=300.0$, $df=1$, $P<0.001$) and *Age* ($F=95.0$, $df=1$, $P<0.001$) effects. Separate analyses for the cerebral cortex (*Age*: $F=67.7$, $df=1$, $P<0.001$) and midbrain (*Age*: $F=32.9$, $df=1$, $P<0.001$) indicated higher binding values at PN75 when compared to PN50 mice for both regions.

The HC-3 binding was also dependent on the region and age (*Region*: $F=49.0$, $df=1$, $P<0.001$; *Age*: $F=10.7$, $df=1$, $P<0.003$; *Region×Age*: $F=6.1$, $df=1$, $P<0.02$). There were no age differences in the cerebral cortex, however, in the midbrain, binding was higher in PN75 when compared to PN50 mice (*Age*: $F=19.6$, $df=1$, $P<0.001$).

Effects on fluid intake/body weight, body weight and brain region weight

There were reductions in fluid intake/body weight for the NIC, ETOH and NIC+ETOH groups when compared to the VEH group, however, these effects were dependent on the day of exposure (*Treatment×Day*: $F=1.5$, $df=25.6$, $P=0.06$). Significant differences were restricted to the first (VEH>all groups; $P=0.001$, FPLSD), fifth (VEH>all groups; $P<0.01$, FPLSD), sixth (VEH>all groups; $P<0.05$, FPLSD) and ninth (VEH>ETOH, VEH>NIC+ETOH, $P<0.01$, FPLSD) days of exposure (Fig. 1A). In spite of treatment differences in fluid intake/body weight, body weight gain (Fig. 1B, 1C) and brain region weight (Table 1) were not affected by treatment (no effect or interactions were observed). These strongly suggest that variations in fluid intake/body weight did not influence the results.

BEC and cotinine levels

The average BEC was highest 30 min after the last (PN44) ethanol injection (137.2±8.5 mg/dl), decreasing considerably 120 min after injection (26.6±5.3 mg/dl). BEC levels did not differ between ETOH and NIC+ETOH mice either 30 min (ETOH: 145.6±13.7 mg/dl; NIC+ETOH: 127.5±8.8 mg/dl) or 120 min (ETOH: 29.9±7.1 mg/dl; NIC+ETOH: 22.7±8.5 mg/dl) after injection.

Table 1. Brain region weights

Measure	VEH	ETOH	NIC	NIC+ETOH
Cerebral cortex	99±6.1	99±6.0	98±7.4	100±5.8
Midbrain	68±5.1	69±6.1	67±8.1	67±6.5

Milligrams.

The cotinine plasma levels did not differ between NIC (122.2±15.5 ng/ml) and NIC+ETOH (133.9±11.27 ng/ml) exposed mice. VEH and ETOH exposed mice presented cotinine levels below the detection limit of the technique (<8 ng/ml).

Overall analysis of cholinergic biomarkers

The global rANOVA, using the one dimensional design (four levels: VEH, ETOH, NIC and NIC+ETOH), across all cholinergic biomarkers, treatments, brain regions, ages and sexes identified interactions of *Treatment*×*Cholinergic Biomarker*×*Region* (F=1.9, df=6, P<0.08) and *Treatment*×*Cholinergic Biomarker*×*Age*×*Sex* (F=1.8, df=6, P<0.09). With the *Ethanol* and *Nicotine* treatments considered as separate factors in a two-dimensional design, we again found interactions indicative of selective effects on each biomarker: *Nicotine*×*Cholinergic Biomarker*×*Region* (F=2.8, df=2, P<0.06) and *Nicotine*×*Cholinergic Biomarker*×*Age*×*Sex* (F=3.6, df=2, P<0.03). In addition, the two dimensions (*Ethanol* and *Nicotine* treatments) interacted with each other in a sex-dependent way: *Ethanol*×*Nicotine*×*Cholinergic Biomarker*×*Sex* (F=2.5, df=2, P=0.09). Considering that *Cholinergic Biomarker* was the only factor present in all interactions described above, which indicated that treatment effects differed among the different biomarkers, data analyses were carried out separately on each biochemical marker and then the results were reexamined. The factors *Brain Region*, *Age* and *Sex* were kept in the analyses. Upon reexamination of data from each biomarker, interactions between *Treatment* and any other factors justified subsequent analyses of separate brain regions, ages or sexes. In the absence of interactions, only the main *Treatment* effect across regions, ages and/or sexes was presented.

Effects on nAChRs ([^{3}H]cytisine binding)

Across both ages, regions and sexes, adolescent treatment elicited a significant *Treatment*×*Age*×*Sex* interaction (F=3.3, df=3, P<0.05) but no interactions of *Treatment* with *Region*. Accordingly, lower-order tests of different regions were not justified. The data were subdivided into separate ages for further analysis. After subdivision, *Sex* was no longer a significant factor.

On PN50 (Fig. 2A, 2C), nAChR upregulation differed among groups (*Treatment* considered as a one-dimensional

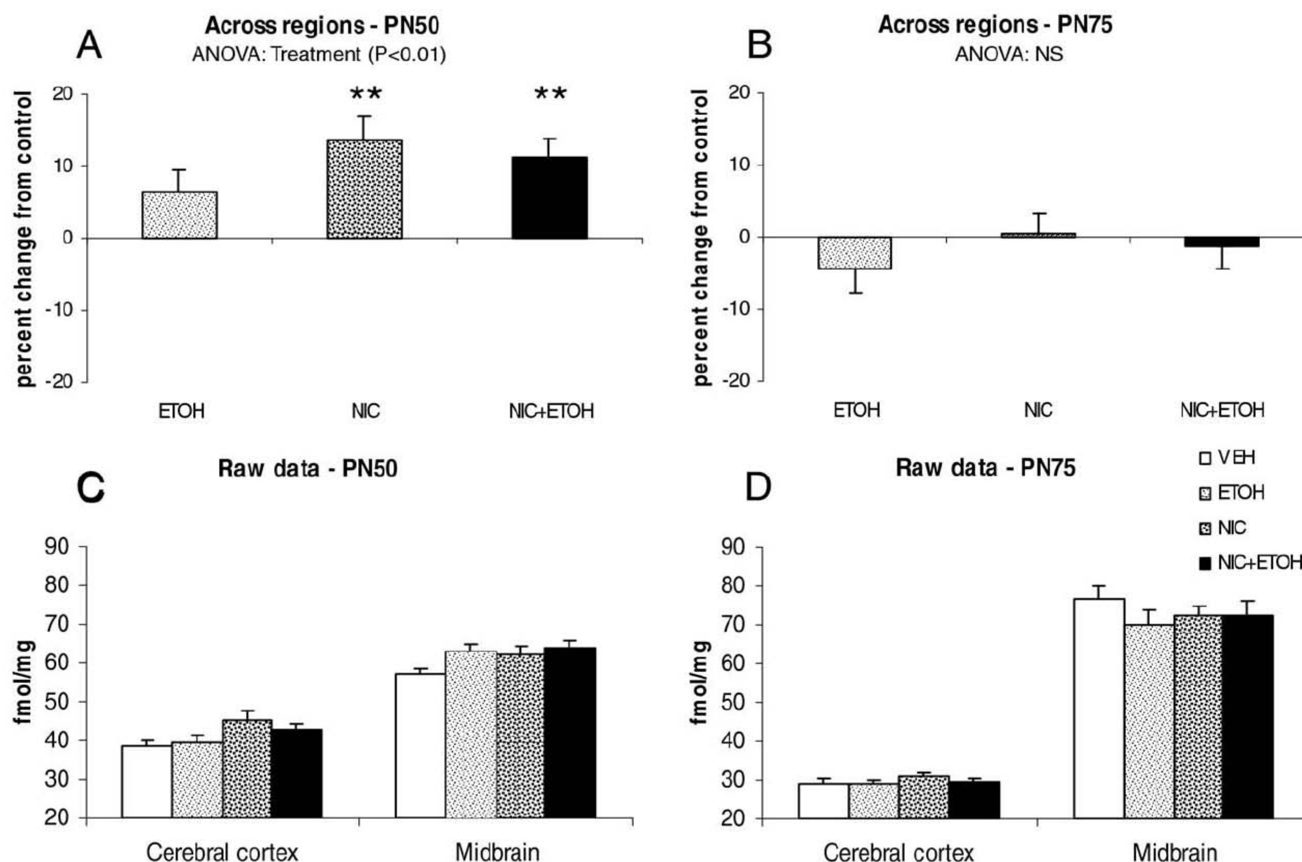

Fig. 2. Effects of adolescent nicotine and/or, ethanol withdrawal on nAChR binding in cerebral cortex and midbrain during short-term (PN50) and long-term (PN75) withdrawal. (A, B) Data are presented as the percent change from control values collapsed across regions. (C, D) Raw data are represented separately for each region. Significant differences between groups as revealed by FPLSD. ** P<0.01 vs. VEH group. Values are means±SEM.

factor—*Treatment*: $F=4.6$, $df=3$, $P<0.01$). Across both regions, ETOH exposure had little or no effect on nAChRs during a short-term withdrawal. However, NIC and the combined NIC+ETOH withdrawal elicited nAChR upregulation when compared to the VEH group ($P<0.01$, FPLSD). Expanding the analysis to consider *Ethanol* and *Nicotine* treatments as two separable factors, the analysis of variance (ANOVA) did not show significant interactions of these factors, connoting the fact that the effects of NIC+ETOH withdrawal were undistinguishable from simple additivity of the effects of nicotine and ethanol.

On PN75 (Fig. 2B, 2D) there were no differences among treatment groups, which indicates that the nAChR upregulation was reversed at long-term withdrawal (no *Treatment* effects or interactions).

Effects on ChAT activity

Across both ages, regions and sexes, adolescent treatment elicited a significant main *Treatment* effect ($F=7.2$, $df=3$, $P=0.001$) as well as a *Treatment*×*Age* interaction ($F=4.8$, $df=3$, $P<0.01$). Accordingly, the data were subdivided into separate ages for further analysis.

Fig. 3 shows results from CHAT activity. Considering the regions together, on PN50, the one-dimensional ANOVA indicated that ChAT activity differed among groups (*Treatment*: $F=6.6$, $df=3$, $P<0.001$). During a short-term withdrawal, ChAT activity was increased in the NIC group when compared to the VEH ($P<0.001$, FPLSD) and ETOH ($P<0.001$, FPLSD) groups. The combined NIC+ETOH exposure also induced an increase in ChAT activity (NIC+ETOH>VEH, $P<0.05$, FPLSD). Expanding the analysis to consider *Nicotine* and *Ethanol* treatments as two separable factors, we verified that ethanol withdrawal reduced the nicotine-induced increase in ChAT activity, revealing a less than additive effect of Nicotine and Ethanol (*Nicotine*×*Ethanol*: $F=4.7$, $df=1$, $P<0.05$).

On PN75, differences between groups (*Treatment*: $F=5.7$, $df=3$, $P<0.01$) reflected a decrease in ChAT activity for the NIC+ETOH group when compared to the VEH ($P<0.01$, FPLSD) and NIC ($P<0.01$, FPLSD) groups. Additionally, NIC long-term withdrawal elicited increased ChAT values when compared to ETOH withdrawal ($P<0.05$, FPLSD). Using the two-dimensional design (*Nicotine* and *Ethanol* treatments considered as separate factors), the

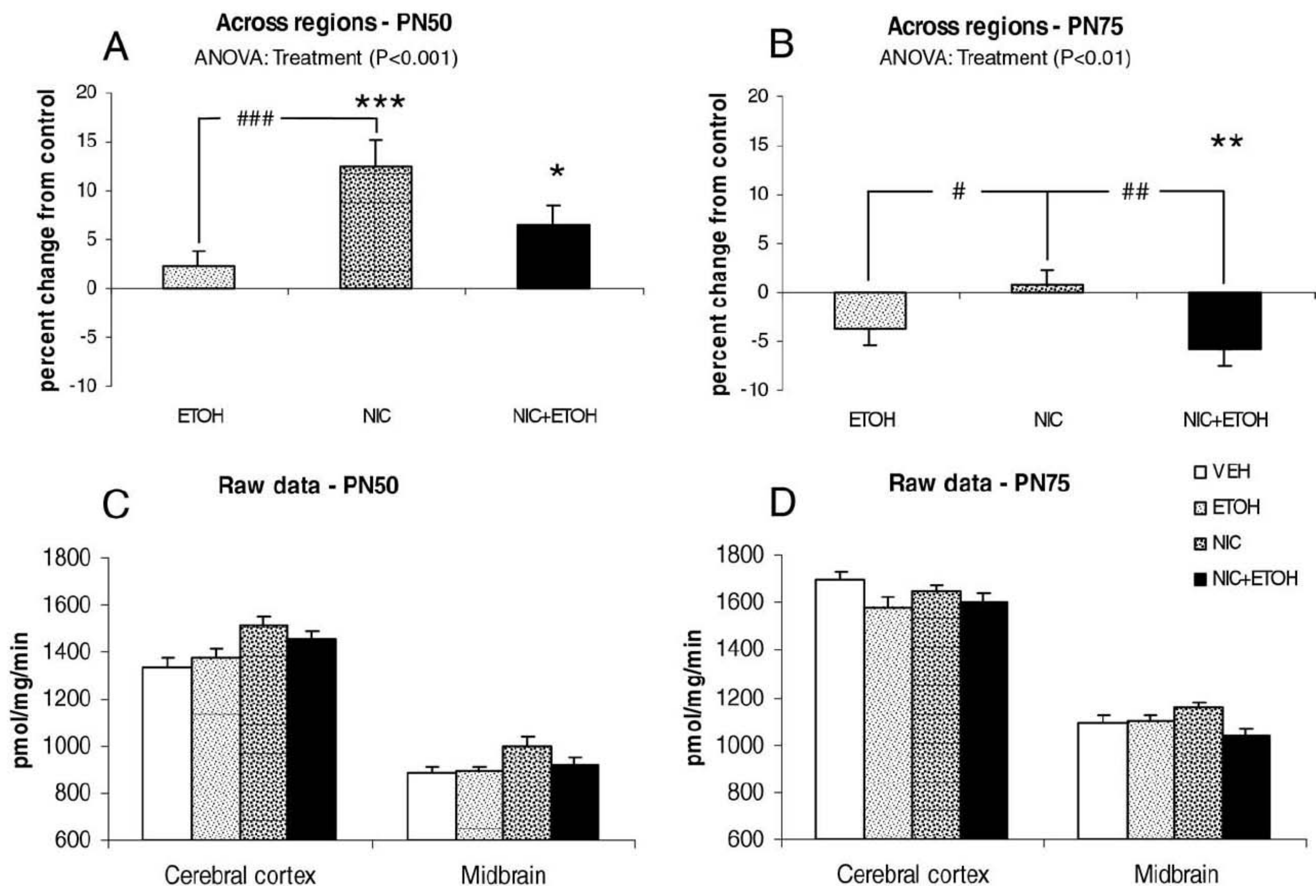

Fig. 3. Effects of adolescent nicotine and/or, ethanol withdrawal on ChAT activity in cerebral cortex and midbrain during short-term (PN50) and long-term (PN75) withdrawal. (A, B) Data are presented as the percent change from control values collapsed across regions. (C, D) Raw data are represented separately for each region. Significant differences between groups as revealed by FPLSD. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. VEH group. # $P<0.05$, ## $P<0.01$, ### $P<0.001$. Values are means±SEM.

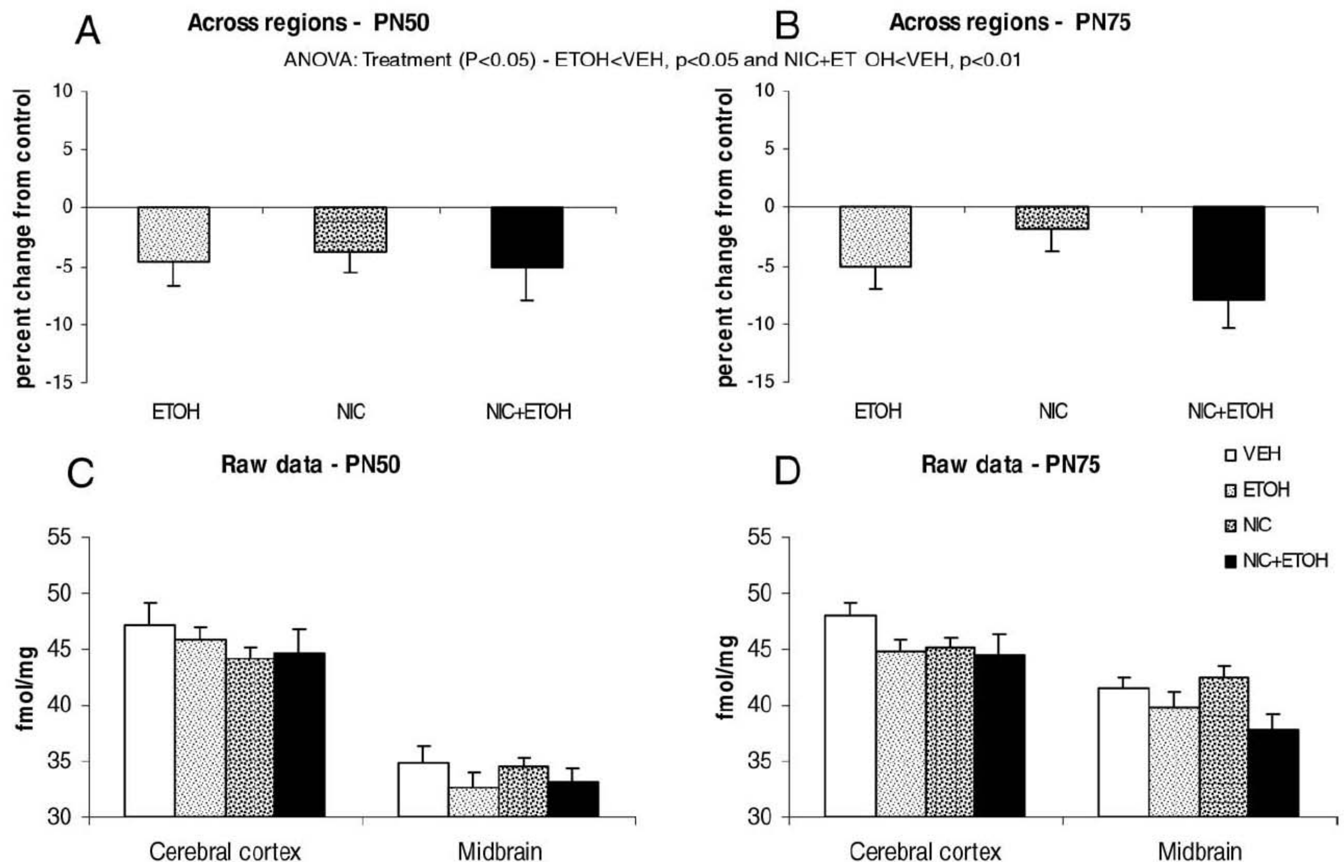

Fig. 4. Effects of adolescent nicotine and/or, ethanol withdrawal on HC-3 binding in cerebral cortex and midbrain during short-term (PN50) and long-term (PN75) withdrawal. (A, B) Data are presented as the percent change from control values collapsed across regions. (C, D) Raw data are represented separately for each region. Values are means±SEM.

ANOVA did not show significant interactions, indicating a simple summation of nicotine and ethanol effects.

Effects on choline transporter ([^{3}H]HC-3 binding)

The ANOVA incorporating all the variables indicated a main *Treatment* effect ($F=3.5$, $df=3$, $P<0.05$), but no interactions of *Treatment* with other variables. Accordingly, lower-order tests of different regions, ages and sexes were not justified. ETOH ($P<0.05$, FPLSD) and combined NIC+ETOH ($P<0.01$, FPLSD) treatment promoted a decrease in HC-3 binding during short- and long-term withdrawal (Fig. 4). The two-dimensional analysis indicated additive effects of nicotine and ethanol (no significant *Nicotine*×*Ethanol* interaction).

DISCUSSION

Results obtained in the current study extend previous findings, which demonstrated that the cholinergic system is a site at which nicotine and ethanol act during adolescence (Ribeiro-Carvalho et al., 2008). Our current findings are consistent with the view that adolescent disruption of the cholinergic function by either nicotine or ethanol is detected even after cessation of drug use, that is, during adolescence and at adulthood. Moreover, our results indicate that, besides their separate effects, these drugs also present interacting effects on the central cholinergic system.

Development of the cholinergic system

In vehicle mice, there were significant changes in the cholinergic biomarkers over the course of development from the end of the adolescence period (PN50) to full adulthood (PN75). In general, our results concerning binding to the $\alpha 4\beta 2$-nAChR subtype, ChAT activity and HC-3 binding are in line with those of previous studies (Happe and Murrin, 1992a; Abreu-Villaça et al., 2003a; Doura et al., 2008), albeit these studies used rats instead of mice. For example, it has been shown that binding to the $\alpha 4\beta 2$-nACh receptor in the cerebral cortex decreases from PN50 to PN75 (Doura et al., 2008). As for ChAT activity (Abreu-Villaça et al., 2003a) and HC-3 binding (Happe and Murrin, 1992a), it has been shown that values increase throughout the period we used in the present study, although it must be pointed out that variations exist between different brain regions. For instance, while ChAT values were previously shown to increase from PN45 to PN65 in rat brain regions

such as the hippocampus, but not in the cerebral cortex and midbrain (Abreu-Villaça et al., 2003a), our results suggest that, in mice, ChAT values increase at least up to PN75 in both cerebral cortex and midbrain. Altogether, our results suggest that the cholinergic system is not fully developed by the end of the period of adolescence since, for most biomarkers and for both regions, developmental changes continue to occur until adulthood.

BEC and cotinine levels

In the present study, in keeping with earlier experimental designs (Abreu-Villaça et al., 2006, 2007, 2008; Manhães et al., 2008; Ribeiro-Carvalho et al., 2008), we chose to give animals free access to a nicotine solution in the drinking water and an injection of ethanol (i.p.) every other day. This experimental design tries to mimic the intermittent pattern of human adolescent drugs consumption. Several studies have been carried out to assess the relation between the pattern of smoking or drinking and indices of addiction (Stratton et al., 2001; Hillemacher et al., 2006). In fact, some studies have even associated the severity of addiction scores with some biomarker levels (Pronko et al., 1997; Rubinstein et al., 2007). In this sense, nicotine and ethanol exposure levels in animal models are important issues in interpreting the results and in strengthening the possibility of generalizations to the human population. Our results indicated that the nicotine concentration used in the drinking solution generated cotinine (nicotine metabolite) plasma levels that are within the range of those found in adolescent smokers (Caraballo et al., 2004; Wood et al., 2004). The ethanol dose used here generated BECs that are within the range that a human adolescent would be exposed to after ingestion of a moderate dose of ethanol (Eckardt et al., 1998). These results show that the experimental design used here and in previous works (Abreu-Villaça et al., 2006, 2007, 2008; Manhães et al., 2008; Ribeiro-Carvalho et al., 2008) can simulate the pattern of nicotine and ethanol exposure observed for human adolescents.

Nicotine and ethanol pharmacokinetic interactions could affect the results of combined exposure. In this regard, it has been demonstrated that ethanol plasma levels are reduced in animals concomitantly exposed to nicotine (Chen and Harle, 2005; Gilbertson and Barron, 2005). However, this effect was only described in animals that received oral ethanol, and seemed to be due to a nicotine-induced delay in gastric emptying (Scott et al., 1993; Chen and Harle, 2005). In the present study, cotinine plasma levels did not differ between NIC- and NIC+ETOH-exposed mice; in the same way, BEC was not affected by nicotine exposure. Our results are in line with those of previous studies, which have demonstrated that i.p. ethanol concentrations are not affected by chronic nicotine exposure (Parnell et al., 2006) and that nicotine exposure does not affect the elimination rate of ethanol (Collins et al., 1988). Taken together, our findings indicate that pharmacokinetic nicotine-ethanol interactions are not capable of explaining our cholinergic results of combined exposure.

Effects on nAChRs

Our previous study (Ribeiro-Carvalho et al., 2008), using a model of free access to a nicotine solution in the drinking water, showed an upregulation effect in the cerebral cortex (13%) and a modest increase in the midbrain (8%). In the present study, we demonstrated that nicotine-induced upregulation was maintained during a short-term period of withdrawal (5 days) and that after a long-term withdrawal (1 month) the upregulation effect was reversed. In general, these results corroborate previous studies in rodents which demonstrated, using an intermittent nicotine exposure paradigm, that the upregulation effect in the midbrain and in the cerebral cortex is still significant 1 week post-treatment, and that nAChR binding tends to return to normal 1 month after the end of nicotine exposure (Abreu-Villaça et al., 2003a). The present results give further support that oral nicotine in the concentration used here is pharmacologically active.

It is well documented that some of the ethanol effects are linked to the function of nAChRs. In this regard, it was demonstrated that ethanol enhancement of dopamine release in the rat nucleus accumbens (Blomqvist et al., 1993) and ethanol-induced locomotor activity (Blomqvist et al., 1992) involve nAChR activation. The $\alpha4\beta2$-nAChR subtype was recently shown to play a role in the modulation of ethanol-induced ataxia in mice (Taslim et al., 2008). In addition, Butt et al. (2004), using three different genetic strategies, suggested that $\alpha4$*-nAChRs modulate ethanol withdrawal. Our previous study (Ribeiro-Carvalho et al., 2008) failed to find effects of ethanol on nAChR binding during the period of exposure. Similarly, in the present study, we demonstrated that ethanol exposure by itself had little or no effect on nAChRs during a short-term or a long-term withdrawal. The short-term treatment and moderate dose used could explain the absence of ethanol effects. For instance, *in vivo* studies using short-term treatment did not detect an effect of ethanol in mice brain nAChRs (Burch et al., 1988; de Fiebre and Collins, 1993).

It has been suggested that ethanol serves as a co-agonist to acetylcholine at some areas of the brain and that the co-exposure could generate a greater susceptibility to trigger nAChR desensitization and, consequently, upregulation. In this regard, it has been suggested that ethanol increases agonist affinity for nAChR (Forman et al., 1989) and modulates nicotine-induced nAChRs upregulation in cell cultures (Dohrman and Reiter, 2003). In general, studies *in vivo* suggest that ethanol enhances the electrophysiological response to nicotine in some but not all brain areas possibly due to variations in the distribution of nAChR subtypes (Breese et al., 1993). In accordance with these data, the combined nicotine+ethanol exposure was recently shown to elicit a robust nAChR upregulation in the cerebral cortex (22%) and the midbrain (26%) of mice (Ribeiro-Carvalho et al., 2008) and that, most importantly, in the midbrain, a brain region that has been associated with mechanisms of reward and addiction (Mansvelder and McGehee, 2002; Nestler, 2001), this upregulation reflected a synergistic effect of nicotine and ethanol (Ribeiro-Car-

valho et al., 2008). To our knowledge, there are no previous studies focusing on cholinergic effects of nicotine+ethanol withdrawal. In the present study, we found that the nicotine+ethanol-elicited upregulation persisted up to five days post-exposure, even though the magnitude of the effects was reduced when compared to the upregulation observed during exposure (Ribeiro-Carvalho et al., 2008). In fact, during the short-term withdrawal, the effects of the combined treatment mostly reflected the summation of nicotine and ethanol alterations both in the cerebral cortex and in the midbrain. Since ethanol failed to elicit nAChR alterations, our present results indicate that, with our experimental design, the cholinergic alterations detected during nicotine+ethanol withdrawal seem to be associated with the previous nicotine exposure. Studies in animal models of adolescent nicotine exposure have shown that nicotine induces its central pharmacological effects by acting on nAChRs, which are ubiquitously distributed in the CNS, mainly at a pre-synaptic level, and serve as ligand-gated ion channels that promote neurotransmitter release (Wonnacott, 1997). Thus, nAChR activation plays a neuromodulatory role in the CNS and is involved in a large number of physiological and pathological processes such as pain neurotransmission, control of movement, cognitive processes, emotional responses, drug abuse and withdrawal (Buisson and Bertrand, 2002; File et al., 2002; Jain, 2004; Jackson et al., 2008; Katner et al., 2004; Schochet et al., 2004). Accordingly, the cholinergic alterations described in the present study may impact one or more of the processes described above. Interestingly, the role of nAChRs in nicotine withdrawal has been recently investigated by Jackson and collaborators (2008): they have suggested that distinct nAChR subtypes play distinct roles in affective and physical signs of nicotine short-term withdrawal. Of particular relevance to our study is their finding that $\beta 2$ knockout mice do not present increased anxiety levels and present loss of aversion in the conditioned place aversion model, which suggests a major role of $\beta 2^*$-nAChRs in affective signs of withdrawal. Since [^{3}H]cytisine binds selectively to the $\alpha 4\beta 2$-nAChR, our results suggest that nicotine-elicited upregulation plays a role in withdrawal events both during nicotine and nicotine+ethanol short-term withdrawal. Normal nAChR binding values at long-term withdrawal suggest that other biological substrates may be more strongly associated with persistent withdrawal events.

In our previous paper (Ribeiro-Carvalho et al., 2008) on the effects of nicotine and/or ethanol during exposure, we demonstrated region-specific alterations in the cholinergic system. In contrast, in the current study, no region-dependent effects were observed. This distinct pattern of results during exposure and withdrawal is, to some extent, puzzling since we were studying the same receptor subtype in both studies and the fact that [^{3}H]cytisine should be labeling $\alpha 4\beta 2$-nAChR almost exclusively. It should be mentioned that nicotine regulates the release of several neurotransmitters, including dopamine, 5-HT, noradrenalin, glutamate, GABA and acetylcholine (Wonnacott, 1997), while ethanol has been shown to enhance function at GABA and glycine receptors, act as a co-agonist at 5-HT receptors, and act as a functional antagonist at glutamate receptors (Larsson and Engel, 2004). It is possible to speculate that region-dependent alterations in these systems might reverberate and interfere, through drug-specific molecular mechanisms, eliciting region-dependent cholinergic alterations. We suggest that, during exposure, these other neurotransmitter systems and the integration of the effects, at synaptic and neural circuit levels, may explain differences in results between brain regions. However, in the absence of the drugs, the role of these systems may be secondary and the direct effects of the drugs in the cholinergic system may predominate. In this regard, fading effects of nicotine (Slotkin and Seidler, 2007) and ethanol (Evrard et al., 2006) were described for the serotonergic system.

Effects on ChAT activity and HC-3 binding

In the cerebral cortex, during adolescent exposure, nicotine was shown to elicit a decrease in ChAT in females (8%) and an increase in males (14%) (Ribeiro-Carvalho et al., 2008). Since ChAT is a constitutive cholinergic synaptic biomarker (Navarro et al., 1989; Happe and Murrin, 1992b; Zahalka et al., 1992, 1993; Aubert et al., 1996), decreased ChAT activity indicates loss of cholinergic innervation and possibly loss of neural cells (Trauth et al., 2000a,b), while elevations in ChAT suggest increased density of cholinergic innervation. Accordingly, these previous results were interpreted as suggestive of cholinergic cell damage in females and sprouting of cholinergic terminals in males. In the present study, ChAT was increased during short-term withdrawal, therefore, for females, the nicotine-elicited cholinergic damage was reversed in the absence of the drug while, for males, the suggested sprouting of terminals elicited during exposure was maintained at least for 5 days after the end of exposure. An increase in the density of cholinergic innervation was previously suggested to represent sprouting as a compensatory response to cell damage (Abreu-Villaça et al., 2003a, 2004a). If this is true, these results suggest that, for males, the recovery from nicotine-elicited effects began still in the presence of the drug and was maintained shortly after exposure. Conversely, females were only able to compensate the cholinergic loss of terminals after the end of exposure. In this sense, several animal studies suggest that females are more sensitive to cell damage due to adolescent nicotine exposure (Trauth et al., 1999, 2000a,b,c; Abreu-Villaça et al., 2003b). At long-term, the elevation in ChAT activity was reduced, which suggests the reestablishment of cholinergic innervation to normal levels. In contrast, nicotine failed to generate alterations in HC-3 binding during short and long term withdrawal. These results indicate that the concentration of synaptic terminals was the major determinant of adolescent nicotine effects on the cholinergic system: We suggest that the increase in ChAT was able to maintain cholinergic synaptic function at normal levels during short-term withdrawal and, at long term, there were no further cholinergic alterations. Nicotine itself during adolescence elicits effects of small magnitude

in ChAT (Abreu-Villaça et al., 2003a; Ribeiro-Carvalho et al., 2008); however, a nicotine-elicited decrease in HC-3 binding was previously shown to occur across several brain regions during exposure and up to 1 month during withdrawal (Abreu-Villaça et al., 2003a). The Abreu-Villaça et al. (2003a) HC-3 results are at odds with our previous findings, during exposure (Ribeiro-Carvalho et al., 2008), and current findings, during withdrawal. These divergent results could be due to differences inherent to the fact that distinct routes of administration have been used. Oral nicotine mimics intermittent exposure characteristic of smokers, which contrasts with other routes such as osmotic minipumps (which produce constant nicotine plasma levels) or injections (which allow for a precise timing of sacrifice relative to the injection) (Abreu-Villaça et al., 2003a).

Our previous study (Ribeiro-Carvalho et al., 2008) found a significant increase (13%) in ChAT in the midbrain. The present results failed to demonstrate ethanol effects in ChAT both during short- and long-term withdrawal. These results suggest that, at least when exposure is restricted to the adolescent period, ChAT returns to normal levels shortly after cessation of exposure. Accordingly, previous studies suggest that more prolonged periods of exposure are necessary to elicit long term cholinergic terminal alterations (Arendt et al., 1988; Casamenti et al., 1993; Floyd et al., 1997). Regarding HC-3, it has been demonstrated that the high affinity choline uptake system is highly resistant to ethanol effects (Saltarelli et al., 1990; Kristofiková et al., 2003; Ribeiro-Carvalho et al., 2008). Here, we demonstrated that despite the lack of effects of ethanol during adolescent exposure (Ribeiro-Carvalho et al., 2008), ethanol-induced HC-3 decrements of small magnitude emerge during a short-term withdrawal and persist at long term. The disparity in ChAT and HC-3 effects suggests a persistent impairment of cholinergic tone in the animals exposed to ethanol. Casamenti and collaborators (1993) demonstrated that 1-week ethanol withdrawal elicited a decrease in acetylcholine release in the cerebral cortex and hippocampus of rats. These results are consistent with the reduced HC-3 binding described in the present study. In addition, the absence of nAChR upregulation described here if associated with a decrease in acetylcholine release reinforces that ethanol elicits cholinergic hypofunction during withdrawal.

Interestingly, despite the fact that nicotine+ethanol combined exposure promoted a small but significant increase in ChAT activity during short-term withdrawal, this effect was less severe than that elicited by nicotine exposure, as indicated by the two-dimensional analysis (less-than-additive effect). A previous study demonstrated less-than-additive effects of nicotine and ethanol on ChAT activity during co-exposure (Ribeiro-Carvalho et al., 2008). Moreover, Penland et al. (2001), addressing cellular neurotoxicity of nicotine and/or ethanol, have also demonstrated less-than-additive effects during short-term withdrawal. The ethanol-elicited reduction of the effect of nicotine suggests two mutually exclusive scenarios. Considering that sprouting of terminals has been suggested to be a compensatory response to cell damage, ethanol co-exposure might protect against nicotine-elicited cell damage. Alternatively, in case the sprouting of terminals is a necessary response to maintain cholinergic function at normal levels, ethanol co-exposure might generate or even intensify a cholinergic impairment. Future studies are necessary to identify the functional relevance of nicotine and ethanol interactions in ChAT activity at short-term withdrawal. At long term withdrawal, despite the lack of effect elicited by either drug, nicotine+ethanol caused a significant decrease in ChAT activity. The loss of cholinergic innervation may be associated with loss of neural cells (Trauth et al., 2000a). Thus, the present study provides direct experimental evidence that the association between nicotine and ethanol exposure promotes long-term loss of cholinergic innervation. To our knowledge, this is the first study that describes the effects of nicotine+ethanol withdrawal in HC-3 binding. In a previous study, the combined exposure failed to demonstrate alterations in HC-3 binding during adolescent exposure (Ribeiro-Carvalho et al., 2008). However, in the present study, a decrease of small magnitude in HC-3 binding was detected during nicotine+ethanol short- and long-term withdrawal. This result suggests a persistent reduction in cholinergic activity elicited by drug withdrawal.

CONCLUSIONS

Tobacco use and ethanol consumption by adolescents are worldwide public health issues of widening severity. Brain development continues into adolescence, being characterized by several modifications that include the maturation of the central cholinergic system (Altman and Bayer, 1990; Zahalka et al., 1993; Rakic et al., 1994). In a recent study, we have demonstrated that the adolescent central cholinergic system is a site at which nicotine and ethanol interact (Ribeiro-Carvalho et al., 2008). Here we show that this system is still a target for nicotine and ethanol effects after cessation of exposure. The magnitude of the cholinergic system alterations described here are small to moderate (up to 12%) and the relevance of the findings is yet to be demonstrated. It is even possible that the effects are adaptive within the setting of withdrawal. However, in conflict with this possibility, functional interactions in the regulation of behavioral responses have been described after the end of adolescent nicotine and ethanol exposure. Particularly, nicotine+ethanol withdrawal was shown to elicit an anxiogenic response (Abreu-Villaça et al., 2008) and an improvement in memory/learning (Abreu-Villaça et al., 2007). Considering the role of the cholinergic system in both memory/learning and anxiety (File et al., 2000; Degroot and Treit, 2002; Hasselmo, 2006; Dani and Bertrand, 2007; Engin and Treit, 2007), the behavioral effects of nicotine+ethanol withdrawal support the assumption that cholinergic system alterations during withdrawal play a role in the physiological consequences of the co-use and abuse.

Acknowledgments—This work was supported by grants from Philip Morris, USA Inc. and Philip Morris International, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-BRAZIL) and by fellowships from Fundação de Amparo à Pes-

quisa do Estado do Rio de Janeiro (FAPERJ-BRAZIL) and Subreitoria de Pós-graduação e Pesquisa da Universidade do Estado do Rio de Janeiro (SR2-UERJ). The authors thank Theodore Slotkin and Frederick Seidler for their help with methodological issues and Edson Oliveira for animal care.

REFERENCES

Abreu-Villaça Y, Medeiros AH, Lima CS, Faria FP, Filgueiras CC, Manhães AC (2007) Combined exposure to nicotine and ethanol in adolescent mice differentially affects memory and learning during exposure and withdrawal. Behav Brain Res 181:136–146.

Abreu-Villaça Y, Nunes F, Queiroz-Gomes FE, Manhães AC, Filgueiras CC (2008) Combined exposure to nicotine and ethanol in adolescent mice differentially affects anxiety levels during exposure, short term and long term withdrawal. Neuropsychopharmacology 33:599–610.

Abreu-Villaça Y, Queiroz-Gomes FE, Dal Monte AP, Filgueiras CC, Manhães AC (2006) Individual differences in novelty-seeking behavior but not in anxiety response to a new environment can predict nicotine consumption in adolescent C57BL/6 mice. Behav Brain Res 167:175–182.

Abreu-Villaça Y, Seidler F, Slotkin TA (2004a) Does prenatal nicotine exposure sensitize the brain to nicotine-induced neurotoxicity in adolescence? Neuropsychopharmacology 29:1440–1450.

Abreu-Villaça Y, Seidler FJ, Tate CA, Cousins MM, Slotkin TA (2004b) Prenatal nicotine exposure alters the response to nicotine administration in adolescence: effects on cholinergic systems during exposure and withdrawal. Neuropsychopharmacology 29:879–890.

Abreu-Villaça Y, Seidler FJ, Qiao D, Tate CA, Cousins MM, Thillai I, Slotkin TA (2003a) Short-term adolescent nicotine exposure has immediate and persistent effects on cholinergic systems: critical periods, patterns of exposure, dose thresholds. Neuropsychopharmacology 28:1935–1949.

Abreu-Villaça Y, Seidler FJ, Tate CA, Slotkin TA (2003b) Nicotine is a neurotoxin in the adolescent brain: critical periods, patterns of exposure, regional selectivity, and dose thresholds for macromolecular alterations. Brain Res 979:114–128.

Altman J, Bayer SA (1990) Migration and distribution of two populations of hippocampal granule cell precursors during the perinatal and postnatal periods. J Comp Neurol 301:365–381.

Arendt T, Henning D, Gray J, Marchbanks R (1988) Loss of neurons in rat basal forebrain projection system after prolonged intake of ethanol. Brain Res Bull 21:563–570.

Aubert I, Cecyre D, Gauthier S, Quirion R (1996) Comparative ontogenic profile of cholinergic markers, including nicotinic and muscarinic receptors, in the rat brain. J Comp Neurol 369:31–55.

Benowitz NL (1992) Cigarette smoking and nicotine addiction. Med Clin North Am 76:415–437.

Blomqvist O, Engel JA, Nissbrandt H, Söderpalm B (1993) The mesolimbic dopamine-activating properties of ethanol are antagonized by mecamylamine. Eur J Pharmacol 249:207–213.

Blomqvist O, Soderpalm B, Engel JA (1992) Ethanol-induced locomotor activity: involvement of central nicotinic acetylcholine receptors? Brain Res Bull 29:173–178.

Bobo JK, Gilchrist LD, Schilling RFN, Noach B, Schinke SP (1987) Cigarette smoking cessation attempts by recovering alcoholics. Addict Behav 12: 209–215.

Breese GR, Morrow AL, Simson PE, Criswell HE, McCown TJ, Duncan GE, Keir WJ (1993) The neuroanatomical specificity of ethanol action on ligand-gated ion channels: a hypothesis. Alcohol Alcohol Suppl 2:309–313.

Buisson B, Bertrand D (2002) Nicotine addiction: the possible role of functional upregulation. Trends Pharmacol 23:130–136.

Burch JB, de Fiebre CM, Marks M, Collins AC (1988) Chronic ethanol or nicotine treatment results in partial cross-tolerance between these agents. Psychopharmacology (Berl) 95:452–458.

Butt CM, King NM, Stitzel JA, Collins AC (2004) Interaction of the nicotinic cholinergic system with ethanol withdrawal. J Pharmacol Exp Ther 308:591–599.

Caraballo RS, Giovino GA, Pechacek TF (2004) Self-reported cigarette smoking vs. serum cotinine among U.S. adolescents. Nicotine Tob Res 6:19–25.

Carmelli D, Swan GE, Robinette D (1993) The relationship between quitting smoking and changes in drinking in World War II veteran twins. J Substance Abuse 5:103–116.

Casamenti F, Scali C, Vannucchi MG, Bartolini L, Pepeu G (1993) Long-term ethanol consumption by rats: effect on acetylcholine release in vivo, choline acetyltransferase activity, and behavior. Neuroscience 56:465–471.

Chen K, Kandel DB (1995) The natural history of drug use from adolescence to the mid-thirties in a general population sample. Am J Public Health 85:41–47.

Chen WJ, Harle LK (2005) Interactive effect of alcohol and nicotine on developing cerebellum: an investigation of the temporal pattern of alcohol and nicotine administration. Alcohol Clin Exp Res 29: 437–442.

Collins AC, Burch JB, de Fiebre CM, Marks MJ (1988) Tolerance to and cross tolerance between ethanol and nicotine. Pharmacol Biochem Behav 29:365–373.

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

Dani JA, Heinemann S (1996) Molecular and cellular aspects of nicotine abuse. Neuron 16:905–908.

De Biasi M, Salas R (2008) Influence of neuronal nicotinic receptors over nicotine addiction and withdrawal. Exp Biol Med (Maywood) 233:917–929.

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

Degroot A, Treit D (2002) Dorsal and ventral hippocampal cholinergic systems modulate anxiety in the plus-maze and shock-probe tests. Brain Res 949:60–70.

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

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

Doura MB, Gold AB, Keller AB, Perry DC (2008) Adult and periadolescent rats differ in expression of nicotinic cholinergic receptor subtypes and in the response of these subtypes to chronic nicotine exposure. Brain Res 1215:40–52.

Eckardt MJ, File SE, Gessa GL, Grant KA, Guerri C, Hoffman PL, Kalant H, Koob GF, Li TK, Tabakoff B (1998) Effects of moderate alcohol consumption on the central nervous system. Alcohol Clin Exp Res 22:998–1040.

Engin E, Treit D (2007) The role of hippocampus in anxiety: intracerebral infusion studies. Behav Pharmacol 18:365–374.

Evrard SG, Duhalde-Vega M, Tagliaferro P, Mirochnic S, Caltana LR, Brusco A (2006) A low chronic ethanol exposure induces morphological changes in the adolescent rat brain that are not fully recovered even after a long abstinence: an immunohistochemical study. Exp Neurol 200:438–459.

File SE, Kenny PJ, Cheeta S (2000) The role of the dorsal hippocampal serotonergic and cholinergic systems in the modulation of anxiety. Pharmacol Biochem Behav 66:65–72.

File SE, Cheeta S, Irvine EE, Tucci S, Akthar M (2002) Conditioned anxiety to nicotine. Psychopharmacology 164:309–317.

Flores CM, Rogers SW, Pabreza LA, Wolfe BB, Kellar KJ (1992) A subtype of nicotinic cholinergic receptor in rat brain is composed of $\alpha 4$ and $\beta 2$ subunits and is upregulated by chronic nicotine treatment. Mol Pharmacol 41:31–37.

Floyd EA, Young-Seigler AC, Ford BD, Reasor JD, Moore EL, Townsel JG, Rucker HK (1997) Chronic ethanol ingestion produces cholinergic hypofunction in rat brain. Alcohol 14:93–98.

Forman SA, Righi DL, Miller KW (1989) Ethanol increases agonist affinity for nicotinic receptors from torpedo. Biochim Biophys Acta 987:95–103.

Friend KB, Pagano ME (2005) Smoking cessation and alcohol consumption in individuals in treatment for alcohol use disorders. J Addict Dis 24:61–75.

Fumagalli F, Jones SR, Caron MG, Seidler FJ, Slotkin TA (1996) Expression of mRNA coding for the serotonin transporter in aged vs. young rat brain: differential effects of glucocorticoids. Brain Res 719:225–228.

Gilbertson RJ, Barron S (2005) Neonatal ethanol and nicotine exposure causes locomotor activity changes in preweanling animals. Pharmacol Biochem Behav 81:54–64.

Grant BF (1998) Age at smoking onset and its association with alcohol consumption and DSM-IV alcohol abuse and dependence: results from the national longitudinal alcohol epidemiologic survey. J Substance Abus 10:59–73.

Grant KA (1994) Emerging neurochemical concepts in the actions of ethanol at ligand-gated ion channels. Behav Pharmacol 5: 383–404.

Gulliver SB, Kamholz BW, Helstrom AW (2006) Smoking cessation and alcohol abstinence: what do the data tell us? Alcohol Res Health 29:208–212.

Happe HK, Murrin LC (1992a) Development of high-affinity choline transport sites in rat forebrain: a quantitative autoradiography study with [^{3}H]hemicholinium-3. J Comp Neurol 321:591–611.

Happe HK, Murrin LC (1992b) High-affinity choline transport regulation by drug administration during postnatal development. J Neurochem 58:2053–2059.

Hasselmo ME (2006) The role of acetylcholine in learning and memory. Curr Opin Neurobiol 16:710–715.

Hillemacher T, Bayerlein K, Wilhelm J, Poleo D, Frieling H, Ziegenbein M, Sperling W, Kornhuber J, Bleich S (2006) Volume intake and craving in alcohol withdrawal. Alcohol Alcohol 41:61–65.

Jackson KJ, Martin BR, Changeux JP, Damaj MI (2008) Differential role of nicotinic acetylcholine receptor subunits in physical and affective nicotine withdrawal signs. J Pharmacol Exp Ther 325:302–312.

Jain KK (2004) Modulators of nicotinic acetylcholine receptors as analgesics. Curr Opin Investig Drugs 5:76–81.

Katner SN, Davis SA, Kirsten AJ, Taffe MA (2004) Effects of nicotine and mecamylamine on cognition in rhesus monkeys. Psychopharmacology 175:225–240.

Klein LC, Stine MM, Pfaff DW, Vandenbergh DJ (2003) Maternal nicotine exposure increases nicotine preference in periadolescent male but not female C57B1/6J mice. Nicotine Tob Res 5:117–124.

Klein LC, Stine MM, Vandenbergh DJ, Whetzel CA, Kamens HM (2004) Sex differences in voluntary oral nicotine consumption by adolescent mice: a dose-response experiment. Pharmacol Biochem Behav 78:13–25.

Klemm N, Kuhar MJ (1979) Post-mortem changes in high affinity choline uptake. J Neurochem 32:1487–1494.

Kristofiková Z, Platilová V, Klaschka J (2003) Age- and sex-dependent effects of ethanol on hippocampal hemicholinium-3 sensitive choline carriers during postnatal development of rats. Neurochem Res 28:397–405.

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

Lau C, Seidler FJ, Cameron AM, Navarro HA, Bell JM, Bartolome J, Slotkin TA (1988) Nutritional influences on adrenal chromaffin cell development: comparison with central neurons. Pediatr Rev 24:583–587.

Manhães AC, Guthierrez MC, Filgueiras CC, Abreu-Villaça Y (2008) Anxiety-like behavior during nicotine withdrawal predict subsequent nicotine consumption in adolescent C57BL/6 mice. Behav Brain Res 193:216–224.

Mansvelder HD, McGehee DS (2002) Cellular and synaptic mechanisms of nicotine addiction. J Neurobiol 53:606–617.

National Institute on Drug Abuse (1998) Nicotine addiction, Washington, DC: NIH Publication Number 98-4342, p 7.

Navarro HA, Seidler FJ, Eylers JP, Baker FE, Dobbins SS, Lappi SE, Slotkin TA (1989) Effects of prenatal nicotine exposure on development of central and peripheral cholinergic neurotransmitter systems. Evidence for cholinergic trophic influences in developing brain. J Pharmacol Exp Ther 251:894–900.

Nelson DE, Giovino GA, Shopland DR, Mowery PD, Mills SL, Eriksen MP (1995) Trends in cigarette smoking among US adolescents, 1974 through 1991. Am J Public Health 85:34–40.

Nestler EJ (2001) Psychogenomics: opportunities for understanding addiction. J Neurosci 21:8324–8327.

Parnell SE, West JR, Chen WJ (2006) Nicotine decreases blood alcohol concentrations in adult rats: a phenomenon potentially related to gastric function. Alcohol Clin Exp Res 30:1408–1413.

Penland S, Hoplight B, Obernier J, Crews FT (2001) Effects of nicotine on ethanol dependence and brain damage. Alcohol 24:45–54.

Piasecki TM, Jorenby DE, Smith SS, Fiore MC, Baker TB (2003) Smoking withdrawal dynamics. I. Abstinence distress in lapsers and abstainers. J Abnorm Psychol 112:3–13.

Pronko PS, Velichko MG, Moroz AR, Rubanovich N (1997) Low-molecular-weight metabolites relevant to ethanol metabolism: correlation with alcohol withdrawal severity and utility for identification of alcoholics. Alcohol Alcohol 32:761–768.

Rakic P, Bourgeois JP, Goldman-Rakic PS (1994) Synaptic development of the cerebral cortex: implications for learning, memory, and mental illness. Prog Brain Res 102:227–243.

Rhodes MC, Seidler FJ, Qiao D, Tate CA, Cousins MM, Thillai I, Slotkin TA (2003) Does pharmacotherapy for preterm labor sensitize the developing brain to environmental neurotoxicants? Cellular and synaptic effects of sequential exposure to terbutaline and chlorpyrifos in neonatal rats. Toxicol Appl Pharmacol 195:203–217.

Ribeiro-Carvalho A, Lima CS, Filgueiras CC, Manhães AC, Abreu-Villaça Y (2008) Nicotine and ethanol interact during adolescence: effects on the central cholinergic systems. Brain Res 1232:48–60.

Rubinstein ML, Thompson PJ, Benowitz NL, Shiffman S, Moscicki AB (2007) Cotinine levels in relation to smoking behavior and addiction in young adolescent smokers. Nicotine Tob Res 9:129–135.

Saltarelli MD, Yamada K, Coyle JT (1990) Phospholipase A2 and (^{3}H)hemicholinium-3 binding sites in rat brain: a potential second-messenger role for fatty acids in the regulation of high-affinity choline uptake. J Neurosci 10:62–72.

Schochet TL, Kelley AE, Landry CF (2004) Differential behavioral effects of nicotine exposure in adolescent and adult rats. Psychopharmacology 175:265–273.

Schorling JB, Gutgesell M, Klas P, Smith D, Keller A (1994) Tobacco, alcohol and other drug use among college students. J Substance Abuse 6:105–115.

Scott AM, Kellow JE, Shuter B, Nolan JM, Hoschl R, Jones MP (1993) Effects of cigarette smoking on solid and liquid intragastric distribution and gastric emptying. Gastroenterology 104:410–416.

Simon JR, Atweh S, Kuhar MJ (1976) Sodium-dependent high affinity choline uptake: a regulatory step in the synthesis of acetylcholine. J Neurochem 26:909–922.

Slotkin TA (2002) Nicotine and the adolescent brain: insights from an animal model. Neurotoxicol Teratol 24:369–384.

Slotkin TA, Seidler FJ (2007) A unique role for striatal serotonergic systems in the withdrawal from adolescent nicotine administration. Neurotoxicol Teratol 29:10–16.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner, FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85.

Snedecor GW, Cochran WG (1967) Statistical methods, 6th ed, p 593 Ames, IA: Iowa State University Press.
Sparks JA, Pauly JR (1999) Effects of continuous oral nicotine administration on brain nicotinic receptors and responsiveness to nicotine in C57B1/6 mice. Psychopharmacology 141:145–153.
Spear L (2000a) Modeling adolescent development and alcohol use in animals. Alcohol Res Health 24:115–123.
Spear LP (2000b) The adolescent brain and age-related behavioral manifestations. Neurosci Biobehav Rev 24:417–463.
Stratton K, Shetty P, Wallace R, Bondurant S (2001) Clearing the smoke: the science base for tobacco harm reduction—executive summary. Tob Control 10:189–195.
Taslim N, Al-Rejaie S, Saeed Dar M (2008) Attenuation of ethanol-induced ataxia by alpha(4)beta(2) nicotinic acetylcholine receptor subtype in mouse cerebellum: a functional interaction. Neuroscience 157:204–213.
Torabi MR, Bailey WJ, Majd-Jabbari M (1993) Cigarette smoking as a predictor of alcohol and other drug use by children and adolescents: evidence of the "gateway drug effect." J Sch Health 63:302–306.
Trauth JA, McCook EC, Seidler FJ, Slotkin TA (2000a) Modeling adolescent nicotine exposure: effects on cholinergic systems in rat brain regions. Brain Res 873:18–25.
Trauth JA, Seidler FJ, Slotkin TA (2000b) An animal model of adolescent nicotine exposure: effects on gene expression and macromolecular constituents in rat brain regions. Brain Res 867:29–39.
Trauth JA, Seidler FJ, Slotkin TA (2000c) Persistent and delayed behavioral changes after nicotine treatment in adolescent rats. Brain Res 880:167–172.
Trauth JA, Seidler FJ, McCook EC, Slotkin TA (1999) Adolescent nicotine exposure causes persistent upregulation of nicotinic cholinergic receptors in rat brain regions. Brain Res 851:9–19.
West RJ, Hajek P, Belcher M (1989) Severity of withdrawal symptoms as a predictor of outcome of an attempt to quit smoking. Psychol Med 19:981–985.
WHO (2004) Global status report on alcohol. World Health Organization, Department of Mental Health and Substance Abuse; http://www.who.int/substance_abuse/publications/global_status_report_2004_overview.pdf. accessed on: june 2009.
Wonnacott S (1997) Presynaptic nicotinic ACh receptors. Trends Neurosci 20:92–98.
Wood T, Wewers ME, Groner J, Ahijevych K (2004) Smoke constituent exposure and smoking topography of adolescent daily cigarette smokers. Nicotine Tob Res 6:853–862.
Zahalka E, Seidler FJ, Lappi SE, Yanai J, Slotkin TA (1993) Differential development of cholinergic nerve terminal markers in rat brain regions: implications for nerve terminal density, impulse activity and specific gene expression. Brain Res 601:221–229.
Zahalka EA, Seidler FJ, Lappi SE, McCook EC, Yanai J, Slotkin TA (1992) Deficits in development of central cholinergic pathways caused by fetal nicotine exposure: differential effects on choline acetyltransferase activity and [^{3}H]hemicholinium-3 binding. Neurotoxicol Teratol 14:375–382.

(Accepted 16 May 2009)
(Available online 22 May 2009)

6. DISCUSSÃO

O tabagismo e o consumo de etanol estão freqüentemente associados (Chen e Kandel, 1995; Torabi et al., 1993; Schorling et al., 1994). Adicionalmente, estudos mostram que o início do hábito de fumar e beber ocorre freqüentemente durante a adolescência, período no qual alterações neuroadaptativas, hormonais e comportamentais ocorrem (Lopez et al., 2001). Apesar dos estudos epidemiológicos, pouco é conhecido sobre os substratos neurobiológicos que regem esta associação, principalmente no período crítico no uso de drogas que é a adolescência. O presente trabalho, representado pelos dois artigos apresentados anteriormente, fornece evidências experimentais que o sistema colinérgico é um denominador comum na interação da nicotina e do etanol na adolescência, demonstrando mecanismos de associação tanto durante a exposição das drogas, como também, no período de abstinência.

6.1. Resumo dos resultados (Tabela 1)

6.1.1. Durante o período de exposição

Ao final do período de exposição (PN45) foi observado que a nicotina promoveu *upregulation* dos nAChRs no córtex cerebral. O mesencéfalo se mostrou menos sensível a esse efeito da nicotina e apresentou apenas um modesto aumento da marcação pela [3H]citisina. A administração do etanol não foi capaz de promover alterações nos nAChRs. Interessantemente, a exposição combinada NIC+ETOH promoveu uma robusta *upregulation* dos nAChRs em ambas as áreas estudadas, destacando-se o efeito sinérgico das drogas sobre o mesencéfalo.

Em relação à atividade da ChAT, os resultados variaram substancialmente em relação as regiões estudadas. A exposição à nicotina resultou em efeitos sexo-dependentes no córtex

cerebral, enquanto que não foi capaz de promover alterações no mesencéfalo. No córtex cerebral, a exposição à nicotina promoveu redução da atividade da ChAT nas fêmeas e aumento da sua atividade nos machos. Já a exposição ao etanol promoveu apenas alteração no mesencéfalo, onde gerou aumento da atividade da ChAT. Surpreendentemente, a exposição combinada das drogas reverteu todos os efeitos gerados por cada droga individualmente, desta forma, não gerando nenhuma diferença para a atividade da ChAT em relação aos controles.

Para os níveis de marcação por HC-3, marcador do transportador pré-sináptico de colina, nenhuma das condições experimentais promoveu alteração ao final do período de exposição.

6.1.2. Durante o período de retirada

Após 5 dias de retirada das drogas (PN50), os animais que foram expostos a nicotina ou nicotina+etanol ainda apresentam a *upregulation* observada durante a exposição, porém, neste caso, animais previamente expostos a NIC+ETOH já não apresentam efeitos significativamente maiores que animais do grupo NIC. Como também foi observado em PN45, a retirada do etanol não foi capaz de promover alteração em PN50. Nenhuma alteração foi observada após 30 dias de retirada das drogas (PN75), indicando que a *upregulation* foi revertida após longo tempo de abstinência.

Em relação a ChAT, a retirada da nicotina promoveu um aumento da sua atividade em PN50. Já a retirada do etanol não gerou nenhuma alteração neste período de curta abstinência. Quando considerada a exposição combinada, apesar do grupo NIC+ETOH apresentar aumento da atividade da ChAT em relação ao controle, este efeito foi estatisticamente menor que o apresentado pelo grupo previamente exposto a nicotina separadamente. Desta forma, a

co-exposição ao etanol parece reverter parcialmente os efeitos gerados pela nicotina na atividade da ChAT após curto tempo de abstinência. Após 30 dias da retirada das drogas, apenas o grupo NIC+ETOH apresentou alteração dos níveis de atividade da ChAT, gerando uma diminuição dessa atividade em relação aos controles.

Em relação ao transportador pré-sináptico de colina, os grupos ETOH e NIC+ETOH apresentaram consistentemente diminuição da ligação do HC-3 ao logo dos períodos de retirada estudados.

Tabela 1 – Resumo dos resultados

		nAChRs	ChAT	HC-3
PN45	NIC	🡅 no CX	🡅 machos no CX 🡇 fêmeas no CX	-
	ETOH	-	🡅 no MS	-
	NIC+ETOH	🡅🡅 no CX 🡅🡅🡅 no MS	-	-
PN50	NIC	🡅🡅	🡅🡅	-
	ETOH	-	-	🡇
	NIC+ETOH	🡅🡅	🡅	🡇
PN75	NIC	-	-	-
	ETOH	-	-	🡇
	NIC+ETOH	-	🡇	🡇

Alterações encontradas pela exposição de nicotina e/ou etanol nos biomarcadores colinérgicos. As setas indicam a direção do efeito (🡅=aumento ou 🡇=diminuição). O número de setas indica a intensidade do efeito (🡅, 🡅🡅, ou 🡅🡅🡅; 🡇, 🡇🡇 ou 🡇🡇🡇) CX, córtex cerebral; MS, Mesencéfalo; PN, pós-natal; nAChR, receptor nicotínico colinérgico; ChAT, colina acetiltransferase; HC-3, hemicolinium.

6.2. Considerações metodológicas

6.2.1 Camundongos C57BL/6

Ratos e algumas cepas de camundongos demonstram aversão ao gosto da nicotina. Além de ser uma das cepas mais usadas para estudos de efeitos comportamentais relacionados à nicotina (Siu e Tyndale, 2007), a cepa C57BL/6 foi escolhida por ser uma cepa que

consome nicotina na concentração utilizada neste estudo. A administração de nicotina via oral tem demonstrado gerar respostas comportamentais (Adriani et al, 2002, 2004; Gaddnas et al, 2001) e alterações neuroquímicas, incluindo a *upregulation* dos receptores nicotínicos (Sparks e Pauly, 1999), alteração nos níveis e metabolismo de monoaminas (Gaddnas et al, 2000; Tammimaki et al, 2006; Vihavainen et al, 2006), assim como alterações na expressão de genes envolvidos na plasticidade sináptica induzidas por drogas de abuso (Marttila et al, 2006) e doença de Alzheimer (Gutala et al, 2006) nesta e em outras cepas. Contudo, a cepa C57BL/6 é conhecida por apresentar características particulares, como alto consumo voluntário de etanol (Belknap et al., 1993; Crawley et al., 1997) o que deve ser levado em consideração em estudos que envolvam a administração desta substância, mesmo que os mecanismos determinantes desta característica específica da cepa não sejam bem conhecidos (Crabbe et al., 1999). Apesar das peculiaridades desta cepa, foram observadas diferenças significativas entre os grupos deste estudo. Estes resultados indicam que existem aspectos fundamentais da neurobiologia destes animais que são afetados pelo tratamento, os quais devem ser mais bem avaliados em futuras investigações. Futuros estudos de bases biológicas de interações entre etanol e nicotina são necessários e o uso de outras cepas e espécies podem fornecer dados valiosos.

6.2.2. Concentração de etanol no sangue e níveis de nicotina

No presente estudo, de acordo com modelos experimentais utilizados recentemente pelo nosso laboratório (Abreu-Villaça et al., 2006, 2007, 2008; Manhães et al., 2008), uma solução de nicotina foi oferecida *ad lib* como a única fonte de líquido, enquanto que injeções i.p. de etanol foram aplicadas a cada 48 horas. Este desenho experimental teve como objetivo mimetizar o padrão de consumo de drogas por humanos adolescentes. Neste sentido, a avaliação do nível de

exposição à nicotina e ao etanol é de fundamental importância para as interpretações dos resultados deste presente estudo, como também, para a possibilidade de generalização com a população humana.

Nossos resultados indicaram que a concentração de nicotina usada nos experimentos gerou níveis plasmáticos de cotinina equivalentes aos encontrados em fumantes adolescentes (Caraballo et al., 2004; Wood et al., 2004). Já a dosagem de etanol usada no presente trabalho promoveu concentração plasmática de etanol média equivalente às encontradas em humanos adolescentes após terem consumido doses consideradas moderadas de etanol (Eckardt et al., 1998). Desta forma, este resultados demonstram que este modelo de exposição usado inclusive em trabalhos anteriores (Abreu-Villaça et al., 2006, 2007, 2008; Manhães et al., 2008) pode simular os níveis de exposição à nicotina e ao etanol observado em adolescentes humanos.

Interações farmacocinéticas entre a nicotina e o etanol poderiam afetar os resultados encontrados devido à exposição combinada no presente trabalho. Em acordo, foi demonstrado que os níveis plasmáticos de etanol são reduzidos em animais expostos concomitantemente a nicotina (Chen e Harle, 2005; Gilbertson e Barron, 2005). Entretanto este efeito só foi demonstrado quando os animais receberam etanol por via oral. Apesar deste fenômeno não ser completamente compreendido, evidências na literatura sugerem que a nicotina reduz a taxa de esvaziamento gástrico, permitindo maior ação da enzima álcool desidrogenase presente no estômago (Scott et al., 1993; Chen e Harle, 2005).

No presente estudo, os níveis de cotinina não diferiram entre os grupos NIC e NIC+ETOH. Da mesma forma, a concentração de etanol no sangue não foi afetada pela exposição à nicotina. Estes resultados estão de acordo com a literatura, que mostra que a concentração do etanol quando administrado i.p. não é afetada pela exposição concomitante da nicotina (Parnell et al., 2006), como também, sugere que a nicotina não afeta a taxa de eliminação do etanol (Collins et al., 1988). Em conjunto, nossos resultados indicam que interações farmacocinéticas entre

nicotina e etanol não são capazes de explicar os resultados encontrados sobre os efeitos da exposição combinada sobre o sistema colinérgico.

6.2.3. Significado e interpretação dos marcadores biológicos

O presente trabalho utilizou para a avaliação do sistema colinérgico ensaios com marcação radioativa para 3 biomarcadores: nAChRs, o transportador pré-sinaptico de colina e a atividade da ChAT.

Para os nAChRs foi utilizado como radioligante a [3H]citisina, que possui alta afinidade pelos receptores do tipo $\alpha4\beta2$. No presente estudo, os ensaios foram realizados utilizando apenas uma concentração de radioligante. Desta forma, a seleção de apenas uma concentração de radioligante para este receptor permite a detecção na mudança de marcação, mas não possibilita estabelecer se este fato se dá por mudança na afinidade do receptor (Kd) ou na sua quantidade (Bmax). Este desenho experimental se faz necessário devido a limitações teciduais e de técnicas intrínsecas ao estudo. Muitas concentrações de radioligante necessariamente envolveriam um maior número de ensaios, tornando necessário um maior volume tecidual. Como o volume tecidual obtido para as áreas estudadas é pequeno, seria necessário processar na mesma amostra tecidos de animais diferentes de um mesmo grupo experimental ("*pool*" de tecido), aumentando, assim, o número total de animais utilizados no estudo. Além disso, para manter padrão ideal de comparação entre os grupos experimentais, o processamento tecidual em cada dia de experimento deverá possuir representantes de todos os grupos de estudo para cada sexo. Se associado a este fato fossem utilizadas várias doses de radioligante, tornaria necessário o processamento de um grande número de amostras em um dia de experimento, tornando-o inviável do ponto de vista técnico. Neste sentido, é importante

considerar que têm sido demonstrado que a *upregulation* dos nAChRs em adolescentes causada pela nicotina representa um aumento do número de receptores sem a mudança do seu grau de afinidade (Trauth et al, 1999).

Além da avaliação dos nAChRs, o presente trabalho também avaliou os efeitos da exposição à nicotina e/ou etanol na atividade da ChAT e na marcação por HC-3, ligante do transportador pré-sináptico de colina de alta afinidade. A ChAT é a enzima responsável pela síntese de acetilcolina. Além disso, tem sido demonstrado que a ChAT aumenta durante o desenvolvimento sinaptico colinérgico, mas não se altera em função de estímulos que aumentam a atividade colinérgica (Aubert et al, 1996; Happe e Murrin, 1992; Navarro et al, 1989; Zahalka et al, 1992, 1993). Desta forma, estes resultados sugerem que esta enzima representa um marcador constitutivo dos terminais colinérgicos, sendo largamente utilizado como uma medida que avalia a magnitude da arborização colinérgica (Abreu-Villaça et al., 2003a; 2004; Qiao et al., 2004; Slotkin et al., 2008). O transportador pré-sinaptico de colina, responsável pela recaptação da colina na fenda sinaptica, tem sido descrito como a etapa tempo limitante para a síntese de acetilcolina (Tucek, 1985; Kuhar e Murrin, 1978). O significado fisiológico deste transportador é demonstrado pelo fato de que o seu bloqueio seletivo pode reduzir a síntese e a liberação de acetilcolina *in vitro* e promover prejuízo da função colinérgica *in vivo* (Happe e Murrin, 1993). Além disso, o transportador de alta afinidade pré-sinaptico de colina é altamente regulado pela atividade neuronal, sendo que esta regulação parece estar relacionada a alteração no número de transportadores existentes. Neste sentido, a medida de marcação do HC-3 tem sido amplamente usada para avaliar a atividade colinérgica neuronal (Abreu-Villaça et al., 2003; 2004; Qiao et al., 2004, Slotkin et al., 2001). Vale mencionar que, deste modo, a comparação entre as mudanças da ChAT e a marcação pelo HC-3 permite a distinção entre os efeitos das drogas estudadas sobre a magnitude da inervação colinérgica e a sua atividade.

6.2.4. Alterações na ingesta e peso

No primeiro estudo (artigo 1), nossos resultados não indicaram nenhuma diferença para a consumo de líquido entre os grupos experimentais. Entretanto no segundo estudo (artigo 2), usando uma amostra maior, demonstramos uma redução no consumo de líquido quando comparamos os grupos tratados com drogas com o grupo veículo. Esta redução foi somente evidenciada em alguns dias de exposição (VEH>todos - 1º, 5º e 6º dias; VEH>NIC e NIC+ETOH – 9º dia). Apesar dessa diferença de consumo apresentada no segundo trabalho, em nenhum dos trabalhos foi evidenciada alteração no peso dos animais. Neste sentido, estes resultados sugerem que as variações no consumo de líquido não influenciaram os resultados bioquímicos apresentados.

6.3. Efeitos sobre o sistema colinérgico

6.3.1. Efeitos sobre nAChRs

Nicotina

É amplamente e consistentemente demonstrado que a exposição à nicotina promove *upregulation* dos nAChRs no cérebro de ratos adultos (Doura et al.,2008; Flores et al., 1992). Estudos anteriores também demonstraram que a exposição à nicotina em ratos adolescentes causa *upregulation* mais robusta e persistente quando comparada com a exposição na idade adulta (Abreu-Villaça et al., 2003a; Doura et al., 2008; Trauth et al., 1999). De forma geral, os resultados do presente trabalho confirmam estudos anteriores e mostram que a nicotina, na

concentração usada foi farmacologicamente ativa: foi evidenciado que a nicotina promoveu *upregulation* no córtex cerebral. Entretanto, a exposição à nicotina só promoveu um modesto aumento da marcação pela [3H]citisina no mesencéfalo. A ausência de resultado no mesencéfalo pode ser explicada pelo modelo de exposição intermitente usado no presente estudo, que poderia gerar alguma recuperação entre cada dose usada pelo animal. De acordo com essa idéia, foi demonstrado que a infusão continua de nicotina promove nAChR *upregulation* mais robusta do que quando a exposição é intermitente (Abreu-Villaça et al., 2003a). Vale ressaltar que também é esperado que a intensidade da *upregulation* gerada pela nicotina varie em função da área estudada (Abreu-Villaça et al., 2003a). Avaliando após a retirada da droga, foi observado que a *upregulation* gerada pela nicotina foi mantida durante um período curto de retirada (5 dias) e que este efeito é totalmente revertido nas áreas estudadas após 1 mês de abstinência. Este resultado também corrobora estudos anteriores, onde foi observado, usando também um padrão intermitente de exposição à nicotina, que a *upregulation* é mantida após uma semana de retirada, mas tende a retornar ao normal após um mês do final da exposição (Abreu-Villaça et al., 2003a).

Etanol

Está bem documentado que alguns efeitos gerados pelo etanol estão ligados a função dos nAChRs. Em acordo, foi demonstrado que o etanol promove liberação de dopamina no núcleo acumbente (Blomqvist et al., 1993) e aumento da atividade locomotora (Blomqvist et al., 1992), sendo ambos fenômenos dependentes da ativação dos nAChRs. Particularmente, o subtipo $\alpha 4\beta 2$ foi recentemente associado com a modulação da geração de ataxia pelo etanol em camundongos (Taslim et al., 2008). Vários estudos têm demonstrado que a exposição ao

etanol pode promover tanto *upregulation* como *downregulation* de nAChR dependendo da região ou da célula utilizada em estudos em cultura (Booker e Collins, 1997; Gorbounova et al., 1998; Robles e Sabriá, 2008). Além disso, Butt e colaboradores (2004), usando três estratégias genéticas, sugerem que α4*-nAChRs modulam a abstinência pelo etanol. No presente trabalho, não foi encontrado efeito do etanol na marcação dos nAChRs durante o período de exposição, como também após a retirada da droga. O pequeno tempo de exposição e a dose moderada usada neste estudo podem explicar a ausência de efeitos do etanol. Em acordo, tem sido demonstrado em estudos *in vivo* que o etanol não gera alterações nos nAChRs em camundongos após pequeno período de exposição (Burch et al., 1988; de Fiebre e Collins, 1993).

Co-exposição

Existem evidências na literatura que sugerem que o etanol funciona como co-agonista da acetilcolina em algumas regiões do cérebro e que a co-exposição à nicotina e etanol poderia gerar uma maior susceptibilidade a dessensibilização dos nAChRs e, conseqüentemente, sua *upregulation*. Em acordo, tem sido sugerido que o etanol aumenta a afinidade dos nAChRs por seus agonistas (Forman et al., 1989) e modula a *upregulation* induzida pela nicotina em cultura de células (Dohrman e Reiter, 2003). O presente trabalho demonstrou que a exposição combinada de nicotina e etanol gerou uma robusta *upregulation* dos nAChRs em ambas as áreas cerebrais estudadas. Vale ressaltar que no mesencéfalo esta *upregulation* reflete um efeito sinergista entre nicotina e etanol. Esse resultado apresentado no mesencéfalo constitui a primeira evidência experimental que a co-administração destas drogas tem um efeito maior que a simples soma dos efeitos individuais de cada droga sobre os

nAChRs. Considerando a importância dos circuitos mesencefálicos nos mecanismos de geração de recompensa e vício (Mansvelder e McGehee, 2002; Nestler, 2001) e o fato de que também se sugere que a *upregulation* dos nAChRs esteja envolvida no estabelecimento desses mecanismos (Buisson e Bertrand, 2002; Dani e De Biasi, 2001; Picciotto et al., 2008), nós sugerimos que, se o mesmo ocorresse em adolescentes humanos, o efeito sinérgico poderia facilitar a geração do abuso de ambas as drogas.

A *upregulation* gerada pela a dupla-exposição persiste até cinco dias após a retirada das drogas, entretanto, a magnitude do efeito observado foi reduzida quando comparada com o período da exposição. De fato, os efeitos da co-exposição refletiram a soma das alterações promovidas individualmente em ambas às áreas estudadas em PN50. Considerando que o etanol não gerou alterações, este resultado indica que as alterações colinérgicas sobre os nAChRs geradas pela co-exposição estão associadas com a exposição prévia a nicotina. Interessantemente, o papel dos nAChRs na abstinência da nicotina foi recentemente investigada por Jackson e colaboradores (2008), onde é sugerido que alguns subtipos de nAChRs participam na geração dos sinais físicos e afetivos da abstinência da nicotina. De particular interesse para o nosso estudo está o achado de que camundongos *knockout* para β2 não apresentam aumento dos níveis de ansiedade, que sugere a importância dos β2*-nAChRs nos sinais afetivos da abstinência. Considerando que a marcação por [3H]citisina é seletiva para os α4β2- nAChRs, nossos resultados sugerem que a *upregulation* induzida pela nicotina é importante tanto na curta abstinência da nicotina, como também, na abstinência da co-exposição. A ausência de resultados sobre os nAChRs após longo período de retirada sugere que outro tipo de substrato biológico pode estar associado com eventos persistentes na abstinência.

6.3.2. Efeitos sobre a ChAT e HC-3

Nicotina

Durante a exposição, a nicotina promoveu alteração na ChAT apenas no córtex cerebral, sendo este efeito sexo-dependente. Nicotina promoveu redução da ChAT nas fêmeas e aumento nos machos. Considerando que a ChAT é um marcador constitutivo da sinapse colinérgica (Aubert et al., 1996; Navarro et al., 1989; Happe e Murrin, 1992; Zahalka et al., 1992, 1993), a redução da ChAT indica perda da inervação colinérgica, podendo refletir morte de neurônios (Trauth et al., 2000). Já o aumento da ChAT sugere aumento da densidade de inervação colinérgica. De fato, enquanto que a ativação dos nAChRs servem como fatores tróficos durante o desenvolvimento (Coronas et al., 2000; Hohmann e Berger-Sweeney, 1998; Navarro et al., 1989; Pugh e Margiotta, 2000), a estimulação colinérgica excessiva altera os padrões de replicação celular, diferenciação e sinaptogênese (Levin e Slotkin, 1998; Slotkin, 2002, 2004), podendo progredir para dano celular (Abreu-Villaça et al., 2003b; Abrous et al., 2002; Slotkin, 2002, 2004). Desta forma, a redução encontrada na ChAT pode refletir a maior susceptibilidade das fêmeas aos efeitos negativos da nicotina. Por exemplo, mulheres fumantes adolescentes possuem um início mais rápido na geração de dependência pela nicotina (DiFranza et al., 2002) e muitos estudos em animais sugerem que fêmeas são mais sensíveis ao dano celular promovido pela exposição à nicotina durante a adolescência (Abreu-Villaça et al., 2003b; Trauth et al., 1999, 2000). Já o aumento da ChAT em machos sugere que a nicotina promove um aumento significativo da inervação colinérgica, a qual pode representar uma resposta compensatória ao dano celular, como sugerido em outros trabalhos (Abreu-Villaça et al., 2003a, 2004), ou poderia ainda ser uma resposta aos efeitos tróficos da

ativação dos nAChRs (Coronas et al., 2000; Hohmann e Berger-Sweeney, 1998; Hohmann et al., 1988; Navarro et al., 1989; Pugh e Margiotta, 2000).

Após curto período de retirada, a nicotina promoveu um aumento da ChAT tanto para machos como para fêmeas. Neste sentido, este resultado sugere que o dano colinérgico gerado durante o período de exposição foi revertido e compensado nas fêmeas, enquanto que nos machos foi mantida a resposta compensatória já observada no final da exposição. Após 30 dias de retirada da nicotina (PN75), a elevação observada em PN50 da atividade da ChAT foi reduzida. Este resultado sugere que ocorre um restabelecimento da inervação colinérgica para o padrão normal.

Quanto ao HC-3, ao contrário da ChAT, a exposição à nicotina não foi capaz de gerar alterações durante o período de exposição ou mesmo após os períodos de retirada estudados. Este resultado sugere que a concentração de terminais colinérgicos foi o principal determinante das alterações colinérgicas geradas pela nicotina. Ao contrário dos resultados encontrados no presente estudo, esperava-se que a exposição à nicotina promovesse redução da marcação por HC-3. Esta expectativa se dava pelo fato de trabalhos anteriores demonstrarem que a nicotina induz redução do HC-3 durante a exposição e que esta redução permanece até um mês da interrupção do tratamento (Abreu-Villaça et al., 2003a). Estes resultados divergentes podem ser resultado de diferentes rotas de administração. A exposição oral à nicotina mimetiza a intermitente exposição característica de fumantes, em contraste com os ratos utilizados no outro estudo, onde minibombas osmóticas (que produzem níveis plasmáticos de nicotina constantes) ou injeções (onde há o controle do tempo entre a última exposição e o momento do sacrifício) foram utilizadas.

Etanol

Ao final do período de exposição, o etanol promoveu apenas alteração na ChAT no mesencéfalo, onde promoveu significante aumento em sua atividade. Estudos anteriores contrariamente mostram que a exposição ao etanol promove diminuição da atividade da ChAT apenas quando o período de exposição é consideravelmente longo (Arendt et al., 1988; Floyd et al., 1997). Uma hipótese possível é que as 48 horas entre as injeções de etanol podem permitir uma resposta compensatória ao possível dano colinérgico no mesencéfalo. O etanol não foi capaz de promover alterações na marcação por HC-3, o que representa que não houve comprometimento da atividade colinérgica pelo etanol durante exposição ao etanol. De forma geral, o transportador de alta afinidade da colina tem se demonstrado altamente resistente aos efeitos do etanol (Kristofiková et al., 2003; Saltarelli et al., 1990), onde apenas altas doses foram capazes de promover alterações.

Em ambos os períodos de retirada estudados o etanol não promoveu nenhuma alteração na atividade da ChAT. Desta forma, o aumento gerado pelo etanol no mesencéfalo foi completamente revertido após curto período de retirada. Em acordo, estudos anteriores sugerem que somente longos períodos de exposição são necessários para promoverem alterações prolongadas na densidade de terminais colinérgicos (Arendt et al., 1988; Casamenti et al., 1993; Floyd et al., 1997). Em relação ao HC-3, apesar da ausência de alterações durante a exposição, nós observamos uma pequena, mas significativa, redução do HC-3 após curto período de retirada que persiste até a vida adulta (PN75). Esta disparidade encontrada entre ChAT e HC-3 sugere um persistente prejuízo na função colinérgica nos animais expostos pelo etanol. De acordo com esta idéia, Casamenti e colaboradores (1993) demonstraram redução da liberação de acetilcolina no córtex cerebral e hipocampo 1 semana após a retirada do etanol em ratos. Além disso, a diminuição da liberação de acetilcolina

juntamente à ausência de *upregulation* descrita neste estudo, reforça a idéia que abstinência do etanol pode gerar hipofunção colinérgica.

Co-exposição

Surpreendentemente, não foram encontradas alterações na atividade da ChAT durante a exposição combinada de nicotina e etanol. No córtex cerebral, apesar de a nicotina promover redução da atividade da ChAT nas fêmeas, a co-exposição não foi capaz de gerar dano na inervação colinérgica, o que sugere um efeito protetor do etanol. Para machos, a co-exposição reverteu o aumento da ChAT provocado pela nicotina. No mesencéfalo, o efeito da co-exposição foi reduzido em relação aos efeitos individuais da nicotina e do etanol, sendo os valores semelhantes aos encontrado no grupo veículo. A co-administração promoveu uma forte *upregulation* dos nAChRs também quando comparado com cada droga individualmente, e como mencionado anteriormente, a excessiva estimulação colinérgica altera o padrão normal do desenvolvimento neuronal (Levin e Slotkin, 1998; Slotkin, 2002, 2004). Entretanto, a exposição combinada no presente estudo não foi capaz de alterar a atividade colinérgica, como indicado pelo resultado da marcação do HC-3. De fato, a investigação da toxicidade da exposição combinada de nicotina e etanol tem se demonstrado controversa. Trabalhos anteriores sobre a toxicidade celular da nicotina/etanol têm mostrado efeitos menos-que-aditivos (Penland et al., 2001; Tizabi et al., 2003, 2004, 2005). Em contraste, Jang e colaboradores (2002) demonstraram que a exposição combinada de nicotina e etanol promove um efeito mais potente quando comparado com a exposição separada de cada droga. Entretanto, recentemente, nosso grupo de pesquisa, usando o mesmo desenho experimental usado aqui, demonstrou que a exposição combinada promoveu efeitos menos severos na

morte celular (Oliveira-da-Silva et al., 2009 *in press*). Apesar dos resultados apresentados nesta tese indicarem que a co-administração não promoveu dano nos terminais colinérgicos, outros tipos celulares podem ser mais afetados. Neste sentido, estudos futuros considerando regiões cerebrais e tipos celulares diferentes devem ser importantes no entendimento dos efeitos gerados pelo co-uso da nicotina e do etanol.

Após curto período de retirada das drogas, foi observado que a exposição combinada promoveu um pequeno, mas significativo, aumento na atividade da ChAT. Assim como apresentado durante a exposição, o efeito da exposição combinada foi menos severo que o provocado pela nicotina. Outros estudos sobre a neurotoxicidade da nicotina e etanol, também têm demonstrado efeitos menos-que-aditivos durante curto período de retirada (Penland et al., 2001). O resultado observado no presente estudo possui duas possibilidades de interpretação. Considerando que o aumento do número de terminais tem sido sugerido como uma resposta compensatória ao dano celular, a exposição ao etanol poderia proteger contra o dano celular gerado pela nicotina. Alternativamente, no caso do aumento do número de terminais ser uma resposta necessária a manutenção da normalidade da função colinérgica, a co-exposição da nicotina com etanol pode promover ou intensificar o prejuízo colinérgico. Estudos futuros são necessários para identificar a relevância funcional da interação entre nicotina e etanol na atividade da ChAT durante curto período de retirada. Após longo período de retirada, apesar de cada droga individualmente não gerar alterações, a exposição combinada gerou diminuição da atividade da ChAT. Considerando que a diminuição da ChAT está associada com a perda de inervação colinérgica, o presente estudo fornece evidencia experimental que a exposição combinada de nicotina e etanol promove perda da inervação colinérgica a longo prazo. Pelo nosso conhecimento, este é o primeiro estudo que avalia os efeitos combinados da nicotina e etanol durante o período de retirada. Apesar de não ocorrer diferenças na marcação por HC-3 durante a exposição das drogas, verificamos uma pequena redução na magnitude da marcação

por HC-3 após a retirada da exposição. Do mesmo modo, foi observada esta redução quando somente foi administrado o etanol. Desta forma, este resultado sugere que a co-exposição gerou redução da atividade colinérgica, muito provavelmente devido à ação da retirada do etanol.

6.4. Possibilidades de outros sítios de interação entre nicotina e etanol

Como mencionado anteriormente, a nicotina regula a liberação de uma grande variedade de neurotransmissores, incluindo dopamina, serotonina, noradrenalina, glutamato GABA e até mesmo a própria acetilcolina (Wonnacott, 1997). Além disso, o etanol diretamente possui ações variadas sobre um grande número de receptores, aumentando a atividade funcional dos receptores do GABA e da glicina, atuando como co-agonista do receptor de serotonina e como antagonista dos receptores de NMDA do glutamato (Larsson e Engel, 2004). Neste sentido, podemos observar que existe uma grande variedade de possíveis alvos de interação entre a nicotina e o etanol, sendo este fato demonstrado em alguns estudos recentes (Al-Rejaie e Dar, 2006a,b; Inoue et al., 2007). O papel de outros sistemas de neurotransmissores e a integração desses efeitos sobre cada região cerebral estudada em particular pode explicar as diferenças de resultados encontrados em alguns pontos neste estudo em cada região cerebral. Estes efeitos integrativos são de grande relevância no entendimento dos mecanismos que regem co-uso, co-abuso e a abstinência da nicotina e do etanol durante a adolescência e, como permanecem incertos, há a necessidade de serem adequadamente avaliados em estudos futuros.

7. CONCLUSÕES

O tabagismo e o consumo do etanol por adolescentes representa um grande problema de saúde pública em todo mundo. O desenvolvimento cerebral continua durante a adolescência, sendo caracterizado por muitas modificações que incluem a maturação do sistema colinérgico central (Altman e Bayer, 1990; Zahalka et al., 1993; Rakic et al., 1994). O entendimento das interações neuroquímicas entre as drogas pode ser essencial no desenvolvimento de novas terapias farmacológicas capazes de reverter os estados de vício, prevenir as recaídas e reduzir o consumo dessas drogas. O presente trabalho indica que o sistema colinérgico central é um denominador comum durante a adolescência para as ações da nicotina e do etanol, como também, é afetado pela retirada das duas drogas.

8. REFERÊNCIAS BIBLIOGRÁFICAS

ABREU VILLAÇA Y *et al.*, Short-term adolescent nicotine exposure has immediate and persistent effects on cholinergic systems: critical periods, patterns of exposure, dose thresholds. Neuropsychopharmacology, 28: 1935-1949, 2003a.

ABREU-VILLAÇA Y *et al.* Combined exposure to nicotine and ethanol in adolescent mice differentially affects memory and learning during exposure and withdrawal. Behav Brain Res., 181: 136–146, 2007.

ABREU-VILLAÇA Y *et al.* Combined exposure to nicotine and ethanol in adolescent mice differentially affects anxiety levels during exposure, short term and long term withdrawal. Neuropsychopharmacology, 33: 599–610, 2008.

ABREU-VILLAÇA Y *et al.* Individual differences in novelty-seeking behavior but not in anxiety response to a new environment can predict nicotine consumption in adolescent C57BL/6 mice. Behaioural Brain Research, 167:175-82, 2006.

ABREU-VILLAÇA Y, SEIDLE FJ, SLOTKIN TA. Nicotine is a neurotoxin in the adolescent brain: critical periods, patterns of exposure, regional selectivity, and dose thresholds for macromolecular alterations. Brain Research, 97: 114-128, 2003b.

ABREU-VILLAÇA Y, SEIDLER F, SLOTKIN TA. Does prenatal nicotine exposure sensitize the brain to nicotine-induced neurotoxicity in adolescence? Neuropsychopharmacology, 29: 1440–1450, 2004.

ABROUS DN *et al.* Nicotine self-administration impairs hippocampal plasticity. J. Neurosci., 22: 3656–3662, 2002.

ADRIANI W *et al.* Behavioral and neurochemical vulnerability during adolescence in mice: studies with nicotine. Neuropsychopharmacology, 29: 869-878, 2004.

ADRIANI W *et al.* Peculiar vulnerability to nicotine oral self-administration in mice during early adolescence. Neuropsychopharmacology, 27:212-224. 2002.

ADRIANI W, CHIAROTTI F, LAVIOLA G. Elevated novelty seeking and peculiar d-amphetamine sensitization in periadolescent mice compared with adult mice. Behavioral Neuroscience , 112: 1152-1166, 1998.

ADRIANI W, LAVIOLA G. A unique hormonal and behavioral hyporesponsivity to both forced novelty and d-amphetamine in periadolescent mice. Neuropharmacology, 39: 334-46, 2000.

ADRIANI W, LAVIOLA G. Elevated levels of impulsivity and reduced place conditioning with d-amphetamine: two behavioral features of adolescence in mice. Behavioral Neuroscience, 117: 695-703, 2003.

ADRIANI W, LAVIOLA G. Windows of vulnerability to psychopathology and therapeutic strategy in the adolescent rodent model. Behavioural Pharmacology, 15: 341-52, 2004.

AGUAYO LG *et al.* GABA(A) receptors as molecular sites of ethanol action. Direct or indirect actions?. Current topics in medicinal chemistry, 2: 869-85, 2002.

ALKONDON M *et al.* Choline and selective antagonists identify two subtypes of nicotinic acetylcholine receptors that modulate GABA release from CA1 interneurons in rat hippocampal slices. The Journal of neurosciense: The official jourrnal of the society for neurosciense. 19: 2693-705, 1999.

ALKONDON M *et al.* Neuronal nicotinic acetylcholine receptor activation modulates gamma-aminobutyric acid release from CA1 neurons of rat hippocampal slices. The Journal of pharmacology and experimental therapeutics, 283:1396-411, 1997.

ALKONDON M *et al.* Nicotinic receptor activation in human cerebral cortical interneurons: a mechanism for inhibition and disinhibition of neuronal networks The Journal of neurosciense: The official jourrnal of the society for neurosciense, 20: 66-75. 2000.

AL-REJAIE S, DAR MS. Antagonism of ethanol ataxia by intracerebellar nicotine: possible modulation by mouse cerebellar nitric oxide and cGMP. Brain Res. Bull., 69: 187–196, 2006a.

AL-REJAIE S, DAR MS. Behavioral interaction between nicotine and ethanol: possible modulation by mouse cerebellar glutamate. Alcohol. Clin. Exp. Res., 30, 1223–1233, 2006b.

ALTMAN J, BAYER SA. Migration and distribution of two populations of hippocampal granule cell precursors during the perinatal and postnatal periods. J. Comp. Neurol., 301: 365–381, 1990.

ANDERSEN SL. Trajectories of brain development: point of vulnerability or window of opportunity? Neuroscience and Biobehavioral Reviews, 27: 3-18, 2003.

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

ARAMAKIS VB, METHERATE R. Nicotine selectively enhances NMDA receptor-mediated synaptic transmission during postnatal development in sensory neocortex. The Journal of neuroscience: the official journal of the society for neuroscience, 18: 8485-95, 1998.

ARENDT T *et al.* Loss of neurons in rat basal forebrain projection system after prolonged intake of ethanol. Brain Res. Bull., 21: 563–570, 1988.

ARNETT JJ. Adolescent storm and stress, reconsidered. The American psychologist, 54: 317-326, 1999.

AUBERT I *et al.* Comparative ontogenic profile of cholinergic markers, including nicotinic and muscarinic receptors, in the rat brain. J. Comp. Neurol., 369, 31–55. 1996.

BALFOUR DJ *et al.* Sensitization of the mesoaccumbens dopamine response to nicotine, Pharmacology Biochemistry and Behavior, 59: 1021–1030, 1998.

BARRON S *et al.* Adolescent vulnerabilities to chronic alcohol or nicotine exposure: findings from rodent models. Alcoholism, Clinical and Experimental Research, 29:1720-5, 2005.

BAYER SA, YACKEL JW, PURI PS. Neurons in the rat dentate gyrus granular layer substantially increase during juvenile and adult life. Science, 216: 890-892, 1982 .

BAYER SA. [3H] Thymidine-radiographic studies of neurogenesis in the rat olfactory bulb. Experimental Brain Research, 50: 329-340, 1983.

BELKNAP JK, CRABBE JC, YOUNG ER. Voluntary consumption of ethanol in 15 inbred mouse strains. Psychopharmacology (Berl), 112: 503-510, 1993.

BENOWITZ NL AND JACOB P 3rd. Metabolism of nicotine to cotinine studied by a dual stable isotope method. Clin Pharmacol Ther, 56: 483-493, 1994.

BENOWITZ NL *et al*. Interindividual variability in the metabolism and cardiovascular effects of nicotine in man. J Pharmacol Exp Ther, 221: 368-372, 1982.

BENOWITZ, NL. Nicotine safety and toxicity. Ed Oxford Univ Press New York, 1998.

BLOMQVIST O *et al.* The mesolimbic dopamine-activating properties of ethanol are antagonized by mecamylamine. Eur. J. Pharmacol., 249: 207–213, 1993.

BLOMQVIST O, SODERPALM B, ENGEL JA. Ethanol-induced locomotor activity: involvement of central nicotinic acetylcholine receptors? Brain Research Bulletin, 29: 173-178, 1992.

BOBO JK *et al*. Cigarette smoking cessation attempts by recovering alcoholics. Addict Behav., 12: 209–215, 1987.

BOOKER TK, COLLINS AC. Long-term ethanol treatment elicits changes in nicotinic receptor binding in only a few brain regions. Alcohol, 14: 131–140, 1997.

BUISSON B, BERTRAND D. Chronic exposure to nicotine upregulates the human (alpha)4((beta)2 nicotinic acetylcholine receptor function. The journal of neuroscience, 21:1819-29, 2001.

BUISSON B, BERTRAND D. Nicotine addiction: the possible role of functional upregulation. Trends in pharmacological sciences, 23:130-6. 2002.

BURCH JB *et al*. Chronic ethanol or nicotine treatment results in partial cross-tolerance between these agents. Psychopharmacology (Berlin), 95: 452–458, 1988.

BURLING TA, ZIFF DC. Tobacco smoking: a comparison between alcohol and drug abuse inpatients. Addictive behaviors, 13: 185-190, 1988.

BUTT CM *et al.* Interaction of the nicotinic cholinergic system with ethanol withdrawal. J Pharmacol Exp Ther., 308: 591–599, 2004.

CARABALLO RS, GIOVINO GA, PECHACEK TF. Self-reported cigarette smoking vs. serum cotinine among U.S. adolescents. Nicotine Tob. Res, 6: 19–25, 2004.

CARMELLI D, SWAN GE, ROBINETTE D. The relationship between quitting smoking and changes in drinking in World War II veteran twins. J Subst Abuse., 5:103-116, 1993.

CARMODY TP *et al.* Co-occurrent use of cigarettes, alcohol, and coffee in healthy, community-living men and women. Health psychology, 4: 323-335, 1985.

CARPENTER-HYLAND EP, CHANDLER LJ. Adaptive plasticity of NMDA receptors and dendritic spines: implications for enhanced vulnerability of the adolescent brain to alcohol addiction. Pharmacol Biochem Behav., 86: 200-208, 2007.

CARR LA, ROWELL PP, PIERCE WM. Effects of subchronic nicotine administration on central dopaminergic mechanisms in the rat. Neurochemical research., 14: 511-515, 1989.

CASAMENTI F *et al*. Long-term ethanol consumption by rats: effect on acetylcholine release in vivo, choline acetyltransferase activity, and behavior. Neuroscience, 56: 465–471, 1993.

CHAN WK, WONG PT, SHEU FS. Frontal cortical alpha7 and alpha4beta2 nicotinic acetylcholine receptors in working and reference memory. Neuropharmacology, 52: 1641-1649, 2007.

CHEN J, MILLAR WJ. Age of smoking initiation: implications for quitting. Health Rep., 9: 39-46, 1998.

CHEN K, KANDEL DB. The natural history of drug use from adolescence to the mid-thirties in a general population sample. American Jouranl of Public Health, 85: 41-47, 1995.

CHEN WJ, HARLE LK. Interactive effect of alcohol and nicotine on developing cerebellum: an investigation of the temporal pattern of alcohol and nicotine administration. Alcohol Clinical Experimental Research, 29: 437-442, 2005**.**

CLARK DB, KIRISCI L, TARTER RE. Adolescent versus adult onset and the development of substance use disorders in males. Drug and alcohol dependence, 49:115-21, 1998.

COLLINS AC *et al.* Tolerance to and cross tolerance between ethanol and nicotine. Pharmacology, Biochemistry and Behavior, 29: 365-373, 1988.

CORONAS V *et al.* Acetylcholine induces neuritic outgrowth in rat primary olfactory bulb cultures. Neurosci., 98: 213–219, 2000.

COURTNEY KE, POLICH J. Binge drinking in young adults: Data, definitions, and determinants. Psychol Bull., 135:142-156, 2009.

COVERNTON PJ, CONNOLLY JG. Differential modulation of rat neuronal nicotinic receptor subtypes by acute application of ethanol. Br J Pharmacol., 122: 1661-1668, 1997.

CRABBE JC *et al.* Identifying genes for alcohol and drug sensitivity: recent progress and future directions. Trends Neurosci., 22: 173–179, 1999.

CRAWLEY JN *et al*. Behavioral phenotypes of inbred mouse strains: implications and recommendations for molecular studies. Psychopharmacology (Berl), 132: 107–124, 1997.

CROWLEY TJ *et al*. Drug and alcohol abuse among psychiatric admissions. A multidrug clinical-tosicologic study. Archives of general psychiatry, 30:13-20, 1974.

DANI JA, DE BIASI M. Cellular mechanisms of nicotine addiction. Pharmacology, Biochemistry, and Behavior, 70: 439-46, 2001.

DANI JA, HARRIS RA. Nicotine addiction and comorbidity with alcohol abuse and mental illness. Nat Neurosci., 8: 1465-1470, 2005.

DANI JA, RADCLIFFE KA, PIDOPLICHKO VI. Variations in desensitization of nicotinic acetylcholine receptors from hippocampus and midbrain dopamine areas. European Journal of Pharmacology, 393:31-8, 2000.

DAR MS, BOWMAN ER, LI C. Intracerebellar nicotinic-cholinergic participation in the cerebellar adenosinergic modulation of ethanol-induced motor incoordination in mice. Brain Res., 644:117-127, 1994.

DE BIASI M. Nicotinic receptor mutant mice in the study of autonomic function. Current Drugs Targets CNS Neurological Disorders, 1:331-6, 2002.

DE FIEBRE CM *et al*. Characterization of a series of anabaseine-derived compounds reveals that the 3-(4)-dimethylaminocinnamylidine derivative is a selective agonist at neuronal nicotinic alpha 7/125I-alpha-bungarotoxin receptor subtypes. Mol Pharmacol., 47: 164-71, 1995.

DE FIEBRE CM, COLLINS AC. A comparison of the development of tolerance to ethanol and cross-tolerance to nicotine after chronic ethanol treatment in long- and short-sleep mice. J Pharmacol Exp Ther., 266:1398-1406, 1993.

DENG XS, DEITRICH RA. Putative role of brain acetaldehyde in ethanol addiction. Curr Drug Abuse Rev., 1:3-8, 2008.

DEYKIN EY, LEVY JC, WELL V. Adolescent depression, alcohol and drug abuse. American journal of Public Health, 77:178-82, 1987.

DI CHIARA G. Role of dopamine in the behavioural actions of nicotine related to addiction. European Journal of Pharmacology, 393:295-314, 2000.

DI FRANZA J *et al*. Development of symptomsof tobacco dependence inyouths: 30-monthfollow-up data from the DANDY study. Tobacco Control, 11: 228–235, 2002.

DI FRANZA JR *et al*. Initial symptoms of nicotine dependence in adolescents. Tobacco control, 9: 313-319, 2000.

DI FRANZA JR, GUERRERA MP. Alcoholism and smoking. Journal of studies on alcohol, 51:130-5, 1990.

DICKSON PA *et al*. Effects of variation at the ALDH2 locus on alcohol metabolism, sensitivity, consumption, and dependence in Europeans. Alcohol Clin Exp Res., 30: 1093–1100, 2006.

DOHRMAN DP, REITER CK. Ethanol modulates nicotine-induced upregulation of nAChRs. Brain Res., 975: 90–98, 2003.

DOMINO EF, YAMAMOTO KI. Nicotine: effect on the sleep cycle of the cat. Science, 150: 637-8, 1965.

DOURA MB *et al*. Adult and periadolescent rats differ in expression of nicotinic cholinergic receptor subtypes and in the response of these subtypes to chronic nicotine exposure. Brain Res., 1215:40–52, 2008.

DUNCAN SC *et al*. Adolescent alcohol use development and young adult outcomes. Drug and alcohol dependence, 49:39-48, 1997.

DUNN AJ, KRAMARCY NR. Neurochemical responses in stress: Relationships between the hypotalamic-pituitary-adrenal and catecholamine systems. In: IVERSEN, LL; IVERSEN SD e SNYDER SH (Eds) Handbook of Psycopharmacology, Vol. 18, New York: Plenum, pp. 455-515, 1984.

DURANCEAUX NC *et al*. Associations of variations in alcohol dehydrogenase genes with the level of response to alcohol in non-Asians. Alcohol Clin Exp Res., 30: 1470–1478, 2006.

ECKARDT MJ *et al.* Effects of moderate alcohol consumption on the central nervous system. Alcoholism, Clinical and Experimental Research, 22:998-1040, 1998.

ELGOYHEN AB *et al*. Alpha 9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. Cell, 79:705-15, 1994.

ELGOYHEN AB *et al*. Alpha10: a determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. Proceedings of the National Academy Science U S A, 98:3501-6, 2001.

ENGEL JA *et al*. In: PALOMO T, BENINGER RJ, ARCHER T, editors. Neurochemical and behavioural studies on ethanol and nicotine interactions. Interactive monoaminergic basis of brain disorders, Madrid. Editorial Sintesis; p. 231-48, 1999.

FENSTER CP *et al*. Upregulation of surface alpha4beta2 nicotinic receptors is initiated by receptor desensitization after chronic exposure to nicotine. The journal of neuroscience, 19:4804-14. 1999.

FERGUSSON DM, LYNSKEY MT, HORWOOD LJ. Childhood exposure to alcohol and adolescent drinking patterns. Addiction, 89:1007-16, 1994.

FILE SE, CHEETA S, KENNY PJ. Neurobiological mechanisms by which nicotine mediates different types of anxiety. European Journal of Pharmacology, 393: 231-6, 2000.

FLORES CM *et al*. A subtype of nicotinic cholinergic receptor in rat brain is composed of a4 and b2 subunits and is upregulated by chronic nicotine treatment. Mol. Pharmacol., 41: 31–37, 1992.

FLOYD EA *et al*. Chronic ethanol ingestion produces cholinergic hypofunction in rat brain. Alcohol, 14: 93–98, 1997.

FORMAN SA, RIGHI DL, MILLER KW. Ethanol increases agonist affinity for nicotinic receptors from Torpedo. Biochim. Biophys. Acta,. 987: 95–103, 1989.

FRIEDMAN J, HUMPHREY JA. Antecedents of collegiate drinking. Journal of youth and adolescence, 14:11-21, 1985.

FRIEND KB, PAGANO ME. Smoking cessation and alcohol consumption in individuals in treatment for alcohol use disorders. J Addict Dis., 24: 61-75, 2005.

FRYE GD *et al*. Effects of acute and chronic ethanol on pre- and postsynaptic responses to baclofen. Brain Res., 560: 84–91, 1991.

FUNK D, MARINELLI PW, LÊ AD. Biological processes underlying co-use of alcohol and nicotine: neuronal mechanisms, cross-tolerance, and genetic factors. Alcohol Res Health., 29:186-192, 2006.

GÄDDNÄS H *et al*. Enhanced motor activity and brain dopamine turnover in mice during long-term nicotine administration in the drinking water. Pharmacol Biochem Behav., 70: 497-503, 2001.

GADDNAS H, PIETILA K, AHTEE L. Effects of chronic oral nicotine treatment and its withdrawal on locomotor activity and brain monoamines in mice. Behav. Brain Res., 13: 65–72, 2001.

GENZEN JR, MCGEHEE DS. Nicotinic modulation of GABAergic synaptic transmission in the spinal cord dorsal horn. Brain research, 1031: 229-37, 2005.

GENZEN JR, MCGEHEE DS. Short and long-term enhancement of excitatory transmission in the spinal cord dorsal horn by nicotinic acetylcholine receptors. Proceedings of the National Academy of Sciences of the United states of America, 100:6807-12, 2003.

GILBERTSON RJ, BARRON S. Neonatal ethanol and nicotine exposure causes locomotor activity changes in preweanling animals. Pharmacology, Biochemistry and Behavior, 81: 54-64, 2005.

GOEDERS NE. A neuroendocrine role in cocaine reinforcement. Psychoneuroendocrinology, 22: 237-59, 1997.

GOPALAKRISHNAN M, MOLINARI EJ, SULLIVAN JP. Regulation of human alpha4beta2 neuronal nicotinic acetylcholine receptors by cholinergic channel ligands and second messenger pathways. Molecular Pharmacology, 52:524-34, 1997.

GORBOUNOVA O *et al*. Chronic ethanol treatment decreases [3H]epibatidine and [3H]nicotine binding and differentially regulates mRNA levels of nicotinic acetylcholine receptor subunits expressed in M10 and SH-SY5Y neuroblastoma cells. J. Neurochem., 70: 1134–1142, 1998.

GOTHONI P, IKOLA W. Ethanol withdrawal tremor potentaates the tremorogenic action of nicotine. Med Biol., 63: 131-136, 1985.

GOTHONI P. Hypersensittvity to the tremorogemc action of nicotine in rats withdrawn from ethanol. Meal Biol., 61: 344- 345, 1983.

GRANT BF, HARFORD TC, GRIGSON MB. Stability of alcohol consumption among youth: A national longitudinal study. Journal of Studies on Alcohol, 49: 253-260, 1987.

GRANT BF. Age at smoking onset and its association with alcohol consumption and DSM-IV alcohol abuse and dependence: results from the National Longitudinal Alcohol Epidemiologic Survey. J Subst Abuse. 10(1):59-73, 1998.

GUTALA R *et al*. Nicotine modulates expression of amyloid precursor protein and amyloid precursor-like protein 2 in mouse brain and in SH-SY5Y neuroblastoma cells. Brain Res., 1093:12-19, 2006.

HANNIGAN JH, ARMANT DR. Alcohol in pregnancy and neonatal outcome. Seminars in Neonatology, 5: 243-54, 2000.

HAPPE HK, MURRIN LC. High-affinity choline transport regulation by drug administration during postnatal development. J. Neurochem., 58, 2053–2059, 1992.

HAPPE HK, MURRIN LC. High-affinity choline transport sites: use of [3H]hemicholinium-3 as a quantitative marker. J Neurochem., 60: 1191-1201, 1993.

HARRIS RA *et al*. Ethanol increases GABAA responses in cells stably transfected with receptor subunits. Alcoholism, Clinical and Experimental Research, 19: 226-32, 1995.

HAWKINS JD *et al*. Exploring the effects of age of alcohol use initiation and psychosocial risk factors on subsequent alcohol misuse. Journal of studies on alcohol, 58:280-90, 1997.

HILL SY *et al*. Factors predicting the onset of adolescent drinking in families at high risk for developing alcoholism. Biological Psychiatry, 48: 265–275, 2000.

HOHMANN CF, BERGER-SWEENEY J. Cholinergic regulation of cortical development and plasticity: new twists to an old story. Perspect. Dev. Neurobiol., 5: 401–425, 1998.

HOHMANN CF, BROOKS AR, COYLE JT. Neonatal lesions of the basal forebrain cholinergic neurons result in abnormal cortical development. Dev. Brain Res., 42: 253–264, 1988.

HUKKANEN J, JACOB P 3rd, BENOWITZ NL. Metabolism and disposition kinetics of nicotine. Pharmacol Rev, 57:79-115, 2005.

HUNT WA. Role of acetaldehyde in the actions of ethanol on the brain - A review. Alcohol, 13: 147–151, 1996.

INOUE Y *et al.* Nicotine and ethanol activate protein kinase A synergistically via G(i) betagamma subunits in nucleus accumbens/ventral tegmental cocultures: the role of dopamine D(1)/D(2) and adenosine A(2A) receptors. J. Pharmacol. Exp. Ther., 322: 23–29, 2007.

INSTITUTO NACIONAL DO CANCER (INCA).Prevalência do tabagismo no Brasil: Dados dos inquéritos epidemiológicos em capitais brasileiras. Rio de Janeiro, 2004.

JACKSON KJ *et al*. Differential role of nicotinic acetylcholine receptor subunits in physical and affective nicotine withdrawal signs. J Pharmacol Exp Ther., 325: 302–312, 2008.

JANG MH *et al*. Nicotine administration decreases nitric oxide synthase expression in the hypothalamus of food-deprived rats. Neuroscience Letters, 322: 29-32, 2002.

JANHUNEN S, AHTEE L. Differential nicotinic regulation of the nigrostriatal and mesolimbic dopaminergic pathways: implications for drug development. Neurosci Biobehav Rev, 313: 287-314, 2007.

JOHNSON RD *et al.* Cigarette smoking and rate of gastric emptying: effect on alcohol absorption. BMJ., 302: 20–23, 1991.

KANDEL DB, LOGAN JA. Patterns of drug use from adolescence to young adulthood: I. Periods of risk for initiation, continued use, and discontinuation. American Journal of Public Health, 74: 660-6, 1984.

KANDEL DB, YAMAGUCHI K, CHEN K. Stages of progression in drug involvement from adolescence to adulthood: further evidence for the gateway theory. J Stud Alcohol, 53:447-457, 1992.

KE L *et al*. Effects of chronic nicotine treatment on expression of diverse nicotinic acetylcholine receptor subtypes. I. Dose- and time-dependent effects of nicotine treatment. J Pharmacol Exp Ther., 286: 825-840, 1998.

KOOB GF *et al*. Neurocircuity targets in ethanol reward and dependence. Alcoholism, Clinical and Experimental Research, 22:3-9, 1998.

KOOB GF. Drugs of abuse: anatomy, pharmacology and function of reward pathways.Trends in pharmacologicals Sciences, 13: 177-84, 1992.

KRISTOFIKOVÁ Z, PLATILOVÁ V, KLASCHKA J. Age- and sex-dependent effects of ethanol on hippocampal hemicholinium-3 sensitive choline carriers during postnatal development of rats. Neurochem. Res., 28: 397–405, 2003.

KUHAR MJ, MURRIN LC. Sodium-dependent, high affinity choline uptake. J Neurochem., 30: 15-21. 1978.

KUNER T, SCHOEPFER R, KORPI ER. Ethanol inhibits glutamate-induced currents in heteromeric NMDA receptor subtypes. Neuroreport, 5: 297–300, 1993.

LALLEMAND F *et al.* Nicotine induced changes of glutamate and arginine in naıve and chronically alcoholized rats: An in vivo microdialysis study. Brain Research, 1111: 48–60, 2006.

LALLEMAND F, WARD RJ, DE WITTE P. Nicotine increases ethanol preference but decreases locomotor activity during the initial stages of chronic ethanol withdrawal. Alcohol Alcohol., 42: 207-218, 2007.

LARANJEIRAS RR, PINSKY I. O alcoolismo. São Paulo: Contexto, 1997.

LARSSON A, ENGEL JA Neurochemical and behavioral studies on ethanol and nicotine interactions. Neuroscience and Biobehavioral Reviews, 27: 713-20, 2004.

LE AD *et al*. Involvement of nicotinic receptors in alcohol self-administration. Alcohol Clinical Exp Research**,** 24: 155-163, 2000.

LEONARD S, BERTRAND D. Neuronal nicotinic receptors: from structure to function.Nicotine & Tobacco Research, 3: 203-23, 2001.

LEVIN ED *et al*. Adolescent-onset nicotine self-administration modeled in female rats. Psychopharmacology (Berl), 169:141-149, 2003.

LEVIN ED, SIMON BB. Nicotinic acetylcholine involvement in cognitive function in animals. Psychopharmacology, 138: 217-30, 1998.

LEVIN ED, SLOTKIN TA. Developmental neurotoxicity of nicotine. In: Slikker, W., Chang, L.W. (Eds.), Handbook of Developmental Neurotoxicology, Academic Press, San Diego, pp. 587–615, 1998.

LEWIS, DA. Development of the prefrontal cortex during adolescence: Insights into vulnerable neural circuits in schizophrenia. Neuropsychopharmacology, 16: 385-398, 1997.

LIMA-LANDMAN MTR, ALBUQUERQUE EX. Ethanol potentiates and blocks NMDA activated single channel currents in rat hippocampal pyramidal cells. FEBS Lett., 247: 61–67, 1989.

LINDSTROM J *et al* Structure and function of neuronal nicotinic acetylcholine receptors. Progress in Brain Research, 109: 125-37, 1996.

LINDSTROM J. Nicotinic acetylcholine receptors in health and disease. Molecular neurobiology, 15: 193-222, 1997.

LITTLE HJ. Behavioral mechanisms underlying the link between smoking and drinking.Alcohol research & health: The journal of the National Institute on Alcohol Abuse and Alcoholism, 24: 215-224, 2000.

LITTLE HJ. The contribution of electrophysiology to knowledge of the acute and chronic effects of ethanol. Pharmacol Ther., 84:333-353, 1999.

LOPEZ MF, WHITE NM, RANDALL CL. Alcohol tolerance and nicotine cross-tolerance in adolescent mice. Addict Biol., 6: 119-127, 2001.

LOVINGER DM, WHITE G, WEIGHT FF. Ethanol inhibits NMDA-activated ion current in hippocampal neurons. Science, 243: 1721-1724, 1989.

LOVINGER DM. 5-HT3 receptors and the neural actions of alcohols: an increasingly exciting topic. Neurochem Int., 35: 125-130, 1999.

LOVINGER DM. Ethanol potentiation of 5-HT3 receptor mediated ion current in NCB-20 neuroblastoma cells. Neurosci Lett., 122, 57–56, 1991.

MACRI'S *et al*. Risk-taking during exploration of a plus-maze is greater in adolescent than in juvenile or adult mice. Animal Behavior, 64: 541-46, 2002.

MAGGS JL, PATRICK ME, FEINSTEIN L. Childhood and adolescent predictors of alcohol use and problems in adolescence and adulthood in the National Child Development Study. Addiction, 103: 7-22, 2008.

MANHÃES AC *et al*. Anxiety-like behavior during nicotine withdrawal predict subsequent nicotine consumption in adolescent C57BL/6 mice. Behav Brain Res., 193:216–224, 2008.

MANSVELDER HD, KEATH JR, MCGEHEE DS. Synaptic mechanisms underlie nicotine-induced excitability of brain reward areas. Neuron, 33: 905-19, 2002

MANSVELDER HD, MCGEHEE D. Cellular and synaptic mechanisms of nicotine addiction. Journal of Neurobiology, 53: 606-617, 2002.

MARKS MJ, BURCH JB, COLLINS AC. Effects of chronic nicotine infusion on tolerance development and nicotinic receptors. J. Pharmacol. Exp. Ther., 226: 817–825, 1983.

MARTTILA K, RAATTAMAA H, AHTEE L. Effects of chronic nicotine administration and its withdrawal on striatal FosB/ DeltaFosB and c-Fos expression in rats and mice. Neuropharmacol., 51: 44–51, 2006.

MARUBIO LM *et al*. Reduced antinociception in mice lacking neuronal nicotinic receptor subunits. Nature, 398:805-10, 1999.

MASOOD K *et al*. Differential ethanol sensitivity of recombinant N-methyl-D-aspartate receptor subunits. Mol Pharmacol., 45: 324–329, 1994.

MATTHEWS DA *et al*. Development of cholinergic innervation in the hippocampal formation of the rat. Histochemical demonstration of acetylcholinesterase activity. Developmental Biology, 36: 130-141, 1974.

MCGEHEE DS, ROLE LW. Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. Annual Review Physiology, 57:521-46, 1995.

MCGINNIS JM, FOEGE WH.. Actual causes of death in the United States. Journal of the American Medical Association, 270: 2207– 2212, 1993.

MIHIC SJ *et al*. Sites of alcohol and volatile anaesthetic action on GABA(A) and glycine receptors. Nature, 389: 385–389, 1997.

MILLER NS, GOLD MS. Comorbid cigarette and alcohol addiction: epidemiology and treatment. J Addict Dis., 17: 55-66, 1998;

MITCHELL SH. Measures of impulsivity in cigarette smokers and non-smokers. Psychopharmacology (Berl), 146: 455-464, 1999.

MOREIRA LB *et al*. Alcoholic beverage consumption and associated factors in Porto Alegre, a southern Brazilian city: a population-based survey. Journal of Studies on Alcoholism, 57: 253-259, 1996.

MORI T *et al*. Basis of variable sensitivities of GABA(A) receptors to ethanol. Alcoholism, Clinical and Experimental Research, 24:965-71, 2000.

MORIGUCHI S *et al*. Effects of ethanol on excitatory and inhibitory synaptic transmission in rat cortical neurons. Alcoholism, Clinical and Experimental Research, 31: 89-99, 2007.

NADLER JV *et al*. Development of cholinergic innervation in the hippocampal formation of the rat. II. Quantitative changes in choline acetyltransferase and acetylcholinesterase activities. Development Biology, 36: 142-154, 1974.

NASHMI R *et al.* Assembly of alpha4beta2 nicotinic acetylcholine receptors assessed with functional fluorescently labeled subunits: effects of localization, trafficking, and nicotine-induced upregulation in clonal mammalian cells and in cultured midbrain neurons, J. Neurosci., 23: 11554–11567, 2003.

NAVARRO HA *et al.* Effects of prenatal nicotine exposure on development of central and peripheral cholinergic neurotransmitter systems. Evidence for cholinergic trophic influences in developing brain. J. Pharmacol. Exp. Ther., 251: 894–900, 1989.

NELSON DA *et al.* Trends in cigarette smoking among US adolescents, 1974 through 1991. American Journal of Public Health, 85:34-40, 1995.

NESTLER EJ. Psychogenomics: opportunities for understanding addiction. J. Neurosci., 21: 8324–8327, 2001.

NOWAK A *et al*. Cigarette smoking delays gastric emptying of a radiolabelled solid food in healthy smokers. Scand J Gastroenterol, 22: 54-58, 1987.

OLIVEIRA-DA-SILVA A *et al*. Increased apoptosis and reduced neuronal and glial densities in the hippocampus due to nicotine and ethanol exposure in adolescent mice. Int J Dev Neurosci., 2009 – *in press*.

OSCARSON M. Genetic polymorphisms in the cytochrome P450 2A6 (CYP2A6) gene: implications for interindividual differences in nicotine metabolism. Drug Metab Dispos, 29:91-95, 2001.

PARNELL SE, WEST JR, CHEN WJ. Nicotine decreases blood alcohol concentrations in adult rats: a phenomenon potentially related to gastric function. Alcohol Clin Exp Res., 30:1408-1413, 2006.

PENLAND S *et al*. Effects of nicotine on ethanol dependence and brain damage. Alcohol, 24: 45–54, 2001.

PERRY DC *et al*. Increased nicotinic receptors in brains from smokers: membrane binding and autoradiography studies, J. Pharmacol. Exp. Ther., 289: 1545–1552, 1999.

PETERSEN, AC; SILBEREISEN, RK; SOERENSON, S. Adolescent development: A global perspective. In: Hurrelmann, K. and Hamilton, S.F. (Eds.) Social Problems and Social Contexts in adolescence: Perspectives Across Boundaries, Hawthorne, NY: Aldine de Gruyter, pp. 3-37, 1996.

PIAZZA PV *et al.* Dopaminergic activity is reduced in the prefrontal cortex and increased in the nucleus accumbens of rats predisposed to develop amphetamine self-administration. Brain Research, 567: 169-74, 1991.

PICCIOTTO MR *et al.* It is not qeither/orq: activation and desensitization of nicotinic acetylcholine receptors both contribute to behaviors related to nicotine addiction and mood. Prog. Neurobiol., 84: 329–342, 2008.

PIERCE JP, GILPIN E. How long will today's new adolescent smoker be addicted to cigarettes? American Journal of Public Health, 86: 253-256, 1996.

POULOS CX, PARKER JL, LE DA. Increased impulsivity after injected alcohol predicts later alcohol consumption in rats: evidence for "loss-of-control drinking" and marked individual differences. Behavioral Neuroscience, 112: 1247-57, 1998.

PRESCOTT CA, KENDLER KS. Age at first drink and risk for alcoholism: a noncausal association. Alcoholism: Clinical and Experimental Research, 23:101-7, 1999.

PUGH PC, MARGIOTTA JF. Nicotinic acetylcholine receptor agonists promote survival and reduce apoptosis of chick ciliary ganglion neurons. Mol. Cell. Neurosci., 15: 113–122, 2000.

QIAO D *et al*. Chlorpyrifos exposure during neurulation: cholinergic synaptic dysfunction and cellular alterations in brain regions at adolescence and adulthood. Brain Res Dev Brain Res., 148: 43-52, 2004.

QUERTEMONT E, TAMBOUR S, TIRELLI E. The role of acetaldehyde in the neurobehavioral effects of ethanol: A comprehensive review of animal studies. Prog Neurobiol., 75:247–274, 2005.

QUICK MW, LESTER RA. Desensitization of neuronal nicotinic receptors. Journal of neurobiology, 53: 457-78, 2002.

RACHAL JV *et al*. Alcohol misure by adolescents. Alcohol Health and Research Would, 6:61-8, 1982.

RAKIC P, BOURGEOIS JP, GOLDMAN-RAKIC PS. Synaptic development of the cerebral cortex: implications for learning, memory, and mental illness. Prog. Brain Res., 102: 227–243, 1994.

REZVANI AH, LEVIN ED. Nicotine-alcohol interactions and cognitive function in rats. Pharmacology, biochemistry and behaviour, 72: 865-872, 2002.

ROBLES N, SABRIÁ J. Effects of moderate chronic ethanol consumption on hippocampal nicotinic receptors and associative learning. Neurobiol. Learn. Mem. 89, 497–503, 2008.

ROLLEMA H *et al*. Rationale, pharmacology and clinical efficacy of partial agonists of alpha4beta2 nACh receptors for smoking cessation. Trends Pharmacol Sci., 28: 316-325, 2007.

ROSS SA *et al*. Phenotypic characterization of an alpha 4 neuronal nicotinic acetylcholine receptor subunit knock-out mouse. The journal of neuroscience: the official journal of the society for neuroscience, 20: 6431-41, 2000.

SALAMONE F, ZHOU M. Aberrations in nicotinic acetylcholine receptors structure, function, and expressions: implications in disease. MJM 5, 90-97, 2000.

SALIN-PASCUAL RJ *et al*. Changes in sleep after acute and repeated administration of nicotine in the rat. Psychopharmacology, 145:133-8, 1999.

SALTARELLI MD, YAMADA K, COYLE JT. Phospholipase A2 and (3H)hemicholinium-3 binding sites in rat brain: a potential second-messenger role for fatty acids in the regulation of high-affinity choline uptake. J. Neurosci., 10: 62–72, 1990.

SAPP DW, YEH HH. Ethanol-GABAA receptor interactions: a comparison between cell lines and cerebellar Purkinje cells. The journal of pharmacology and experimental therapeutics, 284:768-76, 1998.

SARGENT PB. The distribution of neuronal nicotinic acetylcholine receptors. In: Neuronal Nicotinic Receptors (Clement F et al., eds), 163-192, 2000.

SARGENT PB. The diversity of neuronal nicotinic acetylcholine receptors. Annual review of neuroscience, 16: 403-43, 1993.

SCHEPIS TS, ADINOFF B, RAO U. Neurobiological processes in adolescent addictive disorders. Am J Addict., 17: 6-23, 2008.

SCHORLING JB *et al*. Tobacco, alcohol and other drug use among college students. J. Subst. Abuse,.6: 105–115, 1994.

SCHUCKIT MA. Drug and alcohol abuse: a clinical guide to diagnosis and treatment. (6th edn.), Springer, New York, USA, 2006.

SCOTT AM *et al*. Effects of cigarette smoking on solid and liquid intragastric distribution and gastric emptying. Gastroenterology, 104: 410-416, 1993.

SELLERS EM, HIGGINS GA, SOBELL MB. 5-HT and alcohol abuse. Trends Pharmacol Sci., 13: 69–75, 1992.

SILVERI MM, SPEAR LP. Decreased sensitivity to the hypnotic effects of ethanol early in ontogeny: Alcohol Clin Exp Res., 22: 670–676, 1998.

SIU EC, TYNDALE RF. Characterization and comparison of nicotine and cotinine metabolism in vitro and in vivo in DBA/2 and C57BL/6 mice. Mol Pharmacol., 71: 826-834, 2007.

SLAWECKI CJ, THORSELL A, EHLERS CL. Long-term neurobehavioral effects of alcohol or nicotine exposure in adolescent animal models. Annals of the New York Academy of sciences, 1021: 448-52, 2004.

SLAWECKI CJ. Comparison of anxiety-like behavior in adolescent and adult Sprague-Dawley rats. Behavioral Neuroscience, 119: 1477-83, 2005.

SLOTKIN TA *et al*. Adolescent nicotine treatment changes the response of acetylcholine systems to subsequent nicotine administration in adulthood. Brain Res Bull., 76: 152-165, 2008.

SLOTKIN TA *et al*. Perinatal exposure to environmental tobacco smoke upregulates nicotinic cholinergic receptors in monkey brain. Brain Res Dev Brain Res., 133:175-79, 2002.

SLOTKIN TA *et al*. Persistent cholinergic presynaptic deficits after neonatal chlorpyrifos exposure. Brain Res., 902: 229-243, 2001.

SLOTKIN TA, SEIDLER FJ. Nicotine exposure in adolescence alters the response of serotonin systems to nicotine administered subsequently in adulthood. Dev Neurosci, 31:58-70, 2009.

SLOTKIN TA. Cholinergic systems in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates. Toxicol. Appl. Pharmachol., 198: 132–151, 2004.

SLOTKIN, TA. Nicotine and the adolescent brain: insights from an animal model. Neurotoxicology Teratology, 24: 369-384, 2002.

SMITH BR *et al*. Exposure to nicotine enhances acquisition of ethanol drinking by laboratory rats in a limited access paradigm. Pharmacology, 142: 408-412, 1999.

SPARKS JA, PAULY JR. Effects of continuous oral nicotine administration on brain nicotinic receptors and responsiveness to nicotine in C57B1/6 mice. Psychopharmacology, 141: 145–153, 1999.

SPEAR LP, VARLINSKAYA IE. Adolescence. Alcohol sensitivity, tolerance, and intake. Recent developments in alcoholism: an official publication of the American Medical Society on alcoholism, Research Society on Alcoholism and the Nacional Council on Alcoholism, 17:143-59, 2005.

SPEAR LP. The adolescent brain and age-related behavioral manifestations. Neuroscience and Biobehavioral Reviews, 24: 417-63, 2000.

SPEAR LP. The adolescent brain and the college drinker: biological basis of propensity to use and misuse alcohol. Journal of Study Alcohol Supplement, 14: 71-81, 2002.

STOLERMAN IP, SHOAIB M. The neurobiology of tobacco addiction. Trends in pharmacological science, 12: 467-473, 1991.

TAMMIMAKI A *et al.* Effect of quinpirole on striatal dopamine release and locomotor activity in nicotine-treated mice. Eur. J. Pharmacol., 531: 118–125, 2006.

TASLIM N, AL-REJAIE S, SAEED DAR M. Attenuation of ethanolinduced ataxia by alpha(4)beta(2) nicotinic acetylcholine receptor subtype in mouse cerebellum: a functional interaction. Neuroscience, 157: 204–213, 2008.

TASSONYI E *et al.* The role of nicotinic acetylcholine receptors in the mechanisms of anesthesia. Brain Research Bulletin, 57:133-50, 2002.

TEICHER MH *et al.* The neurobiological consequences of early stress and childhood maltreatment. Neuroscience and Biobehavioral Reviews , 27: 33-44, 2003.

TIZABI Y *et al*. Nicotine inhibits ethanol-induced tocixity in cultured cerebral cortical cells. Neurotox. Res., 6: 311–316, 2004.

TIZABI Y *et al.* Protective effects of nicotine on ethanol-induced toxicity in cultured cerebellar granule cells. Neurotox. Res., 5: 315–321, 2003.

TIZABI Y, MANAYE KF, TAYLOR RE. Nicotine blocks ethanol-induced apoptosis in primary cultures of rat cerebral cortical and cerebellar granule cells. Neurotox. Res., 7: 319–322, 2005.

TORABI MR, BAILEY, WJ, MAJD-JABBARI, M. Cigarette smoking as a predictor of alcohol and other drug use by children and adolescents: evidence of the 'Gateway Drug Effect'. J. Sch. Health, 63: 302–306, 1993.

TRACY HA, WAYNER MJ, ARMSTRONG DL. Nicotine blocks ethanol and diazepam impairment of air righting and ethanol impairment of maze performance. Alcohol, 18:123-130, 1999.

TRAUTH JA *et al*. Adolescent nicotine exposure causes persistent upregulation of nicotinic cholinergic receptors in rat brain regions. Brain research, 851: 9-19, 1999.

TRAUTH JA *et al*. Modeling adolescent nicotine exposure: effects on cholinergic systems in rat brain regions. Brain Res., 873: 18–25, 2000.

TSAI G, COYLE JT. The role of glutamatergic neurotransmission in the pathophysiology of alcoholism. Annual Review of Medicine. 49:173-84, 1998.

TUCEK S. Regulation of acetylcholine synthesis in the brain. J Neurochem., 44: 11-24, 1985.

TUGAY M *et al*. Impaired gastric motility in the gastroesophageal reflux rat model: an in vitro study. J Surg Res., 115: 272–278, 2003.

TUPALA E *et al*. Dopamine D(2)/D(3)-receptor and transporter densities in nucleus accumbens and amygdala of type 1 and 2 alcoholics. Molecular psychiatry, 6:261-7, 2001.

URAKAWA N *et al*. Simultaneous determination of nicotine and cotinine in various human tissues using capillary gas chromatography/mass spectrometry. Int J Legal Med, 106: 232-236, 1994.

VASTOLA BJ *et al*. Nicotine induced conditioned place preference in adolescent and adult rats. Physiol Behav., 77: 107–114, 2002.

VIHAVAINEN T *et al*. Effect of morphine on locomotor activity and striatal monoamine metabolism in nicotine-withdrawn mice. Behav. Brain Res., 173: 85–93, 2006.

VOLKOW ND *et al*. Decreases in dopamine receptors but not in dopamine transporters in alcoholics. Alcoholism, clinical and experimental research. 20: 1594-8, 1996.

VOLKOW ND, LI TK. Drugs and alcohol: treating and preventive abuse, addiction and their medical consequences. Pharmacology and therapeutics, 108: 3-17, 2005.

WAFFORD KA *et al*. Ethanol sensitivity of the GABAA receptor expressed in Xenopus oocytes requires 8 amino acids contained in the gamma 2L subunit. Neuron, 7:27-33, 1991.

WAFFORD KA *et al*. Genetic differences in the ethanol sensitivity of GABAA receptors expressed in Xenopus oocytes. Science, 249: 291-3, 1990.

WAFFORD KA, WHITING PJ. Ethanol potentiation of GABAA receptors requires phosphorylation of the alternatively spliced variant of the gamma 2 subunit. FEBS letters, 313: 113-7, 1992.

WALLACE JR JM *et al*. Gender and ethnic differences in smoking, drinking illict drug use among American 8 th, 10th and 12 th grade students, 1976-2000. Addiction, 98: 225-34, 2003.

WANG H, SUN X. Desensitized nicotinic receptors in brain. Brain research, 48:420-37, 2005.

WEBSTER RA, HUNTER M, KEATS JA. Peer and parental influences on adolescents' substance use: a path analysis. The international journal of the Addictions, 29: 647-57, 1994.

WHITE AM *et al*. Chronic-intermittent ethanol exposure during adolescence prevents normal developmental changes in sensitivity to ethanol-induced motor impairments. Alcoholism, Clinical and Experimental Research, 26: 960-968, 2002.

WHITE AM, SWARTZWELDER HS. Hippocampal function during adolescence: a unique target of ethanol effects. Annals New York Academic Science, 1021: 206-220, 2004.

WHITING P, LINDSTROM J. Purification and characterization of a nicotinic acetylcholine receptor from rat brain. Proc Natl Acad Sci., 84: 595–599, 1987.

WHITING PR, LINDSTROM J. Characterization of bovine and human neuronal nicotinic acetylcholine receptors using monoclonal antibodies. J Neurosci., 8: 3395–3404, 1988.

WISE RA, ROMPRE PP. Brain dopamine and reward. Annual review of psychology. 40:191-225, 1989.

WONNACOTT S, SIDHPURA N, BALFOUR DJ. Nicotine: from molecular mechanisms to behaviour. Current opinion in pharmacology, 5:53-9, 2005.

WONNACOTT S. Presynaptic nicotinic ACh receptors. Trends in neurosciences, 20: 92-8, 1997.

WOOD T *et al*. Smoke constituent exposure and smoking topography of adolescent daily cigarette smokers. Nicotine Tob. Res. 6, 853–862, 2004.

WORLD HEALTH ORGANIZATION (WHO) – CONVENÇÃO QUADRO DE CONTROLE DO TABACO. Dia Mundial Sem Tabaco. Tabaco e Pobreza – Um Círculo Vicioso. 31 de maio de 2004.

WORLD HEALTH ORGANIZATION (WHO) FRAMEWORK CONVENTION TOBACCO CONTROL. A mortalidade tabágica mundial em 2002 foi de 4,9 milhões, com os dados recentes provindos dos países em desenvolvimento. Genebra, Janeiro, 2003.

WU G, TONNER PH, MILLER KW. Ethanol stabilizes the open chennel stat of the Torpedo nicotinic acetylcholine receptor. Mol Pharmacol., 45:102–108, 1993.

WÜLLNER U *et al*. Smoking upregulates alpha4beta2* nicotinic acetylcholine receptors in the human brain. Neurosci Lett., 430: 34-37, 2008.

XIAO C *et al*. Nicotine modulates GABAergic transmission to dopaminergic neurons in substantia nigra pars compacta. Acta Pharmacol Sin, 30: 851-8, 2009.

XU W *et al*. Megacystis, mydriasis, and ion channel defect in mice lacking the alpha3 neuronal nicotinic acetylcholine receptor. Proceedings of the national academy of science of the USA, 96: 5746-511, 1999a.

XU W *et al*. Multiorgan autonomic dysfunction in mice lacking the beta2 and the beta4 subunits of neuronal nicotinic acetylcholine receptors. The journal of neuroscience: the official journal of the society for neuroscience, 19: 9298-305, 1999b.

YANG X *et al*. Evidence for a selective effect of ethanol on N-methyl-D-aspartate responses: ethanol affects a subtype of the ifenprodil-sensitive N-methyl- D-aspartate receptors. J Pharmacol Exp Ther., 278: 114–124, 1996.

YTTRI EA, BURK JÁ, HUNT PS Intermittent ethanol exposure in adolescent rats: dose-dependent impairments in trace conditioning. Alcoholism, Clinical and Experimental Research, 28:1433-6, 2004.

YU D *et al*. Ethanol inhibition of nicotinic acetylcholine type alpha 7 receptors involves the amino-terminal domain of the receptor. Mol. Pharmacol., 50: 1010–1016, 1996.

ZAHALKA E *et al*. Differential development of cholinergic nerve terminal markers in rat brain regions: Implications for nerve terminal density, impulse activity and specific gene expression. Brain Research, 601: 221-229, 1993.

ZAHALKA EA *et al*. Deficits in development of central cholinergic pathways caused by fetal nicotine exposure: differential effects on choline acetyltransferase activity and [3H]hemicholinium-3 binding. Neurotoxicol Teratol., 14: 375–382, 1992.

ZHAO R, CHEN H, SHARP BM. Nicotine-induced norepinephrine release in hypothalamic paraventricular nucleus and amygdala is mediated by N-methyl-D-aspartate receptors and nitric oxide in the nucleus tractus solitarius. J Pharmacol Exp Ther., 320:837-44, 2007.

ZUCKERMAN M. Smoking, drinking, drugs and eating. In: Behavioral Expressions and Biosocial Basis of Sensation Seeking. Cambridge, United Kingdom: Cambridge University Press, 225-257, 1994.

Livros Grátis

(http://www.livrosgratis.com.br)

Milhares de Livros para Download:

Baixar livros de Administração
Baixar livros de Agronomia
Baixar livros de Arquitetura
Baixar livros de Artes
Baixar livros de Astronomia
Baixar livros de Biologia Geral
Baixar livros de Ciência da Computação
Baixar livros de Ciência da Informação
Baixar livros de Ciência Política
Baixar livros de Ciências da Saúde
Baixar livros de Comunicação
Baixar livros do Conselho Nacional de Educação - CNE
Baixar livros de Defesa civil
Baixar livros de Direito
Baixar livros de Direitos humanos
Baixar livros de Economia
Baixar livros de Economia Doméstica
Baixar livros de Educação
Baixar livros de Educação - Trânsito
Baixar livros de Educação Física
Baixar livros de Engenharia Aeroespacial
Baixar livros de Farmácia
Baixar livros de Filosofia
Baixar livros de Física
Baixar livros de Geociências
Baixar livros de Geografia
Baixar livros de História
Baixar livros de Línguas

Baixar livros de Literatura
Baixar livros de Literatura de Cordel
Baixar livros de Literatura Infantil
Baixar livros de Matemática
Baixar livros de Medicina
Baixar livros de Medicina Veterinária
Baixar livros de Meio Ambiente
Baixar livros de Meteorologia
Baixar Monografias e TCC
Baixar livros Multidisciplinar
Baixar livros de Música
Baixar livros de Psicologia
Baixar livros de Química
Baixar livros de Saúde Coletiva
Baixar livros de Serviço Social
Baixar livros de Sociologia
Baixar livros de Teologia
Baixar livros de Trabalho
Baixar livros de Turismo