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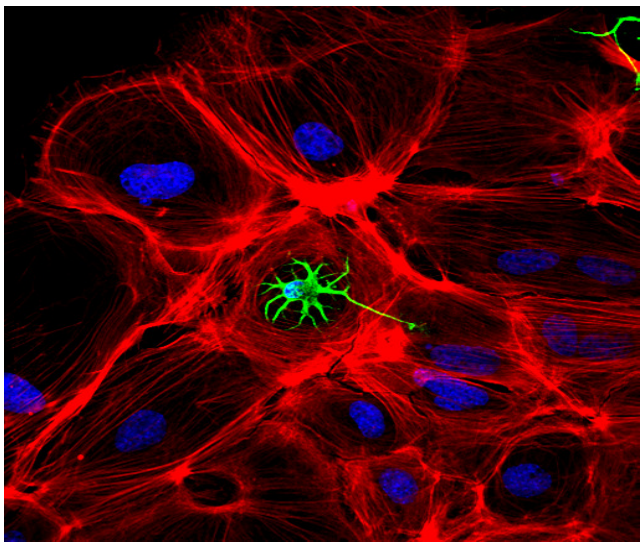

Susanna Molas Casacuberta

TESI DOCTORAL UPF / ANY 2012

DIRECTORA DE LA TESI

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Neurobehavioral Phenotyping of Mouse Models of Disease- Genes and Disease Programme- Centre for Genomic Regulation (CRG)

DEPARTMENT CEXS UPF – PHD PROGRAMME IN BIOMEDICINE

UNIVERSITAT POMPEU FABRA

CRG Centre for Genomic Regulation

Als meus pares, i a la Moni i la Cris,
per estar sempre al meu costat

El temps no s'aprofita,

el temps s'ha de viure.

(R. Panikkar, 1918-2010)

Endinsar-se al món de la ciència és fàcil, captivador, pensar com dissenyar el què, per tal de, i llavors què més... Mantenir-se al món de la ciència ja és una altra cosa. Ara, que veig com aquesta etapa es va tancant, m'adono que en realitat la meva vivència durant els últims anys ha transcorregut de forma molt similar a una de les meves carreres esportives. I en aquest punt, m'identifico en moltes paraules del Dr. Murakami. L'emoció del tret d'inici, l'energia que sembla ser inesgotable, la lleugeresa, fan que els primers kilòmetres passin ràpidament. Ara bé, mantenir el ritme implica exigència, esforç i sobrepassar un immens desgast. Alguns kilòmetres es fan durs, inclús sembla que l'aire costi de respirar, però en altres tornes a recuperar l'alè, l'energia, i vas continuant a bon ritme, fins que es va acostant el final, i llavors, ja queda poc per arribar a meta.

He tingut la sort que durant tot aquest recorregut m'han acompanyat i donat forces els amics i la família, estic segura que sense ells, no hauria arribat on sóc, i per tant es mereixen que els dongui les gràcies.

Primerament, vull agrair a la Dra. Mara Dierssen, cap de grup del laboratori de fenotipació neuroconductual de models murins de malaltia, per haver-me donat la oportunitat d'iniciar-me al món de la ciència. Mara, moltes gràcies per el teu recolzament durant aquests anys. També agrair al Dr. Xavier Estivill, cap del programa de Gens i Malaltia, i als companys dels laboratoris veïns, de la Susanna de la Luna, la Cristina Fillat i la Mariona Arabonés, amb qui moltes vegades hem intercanviat dubtes i productes del laboratori i molts bons moments. Gràcies també al servei de microscopia del CRG, i al servei d'estabulari del PRBB.

Treballar al laboratori de la Mara no només ha consistit en compartir dubtes de poiata; el record que m'enduc va molt més enllà. No puc evitar remuntar als meus inicis, ara ja fa ben bé uns quatre anys:

La meva arribada al laboratori va coincidir amb la de la **Mónica Santos**. Al cap de dos mesos, marxàvem les dues juntes cap al Cajal Winter Conference de Benasque, d'on en recordo un viatge de corbes perdudes enmig de les muntanyes, un curs d'iniciació a snowboard i moltes rialles. D'aquell viatge en va començar una bona amistat, que es va reforçar més endavant a Tarragona, i a Sicília (sí, sempre que jo no faci de copilot.. ;P).

Amb ella he disfrutat d'hores i hores de sol a la platja, i a la terrassa, entre converses i consells. Mónica, gràcies per la teva confiança, ja saps que t'aprecio com una germana, i que et desitjo tota la felicitat del món.

En **Xavi Gallego** va ser qui em va ensenyar sobre els receptors de nicotina i el model TgCHRNA5/A3/B4. Recordo l'estrès dels experiments d'AMP cíclic, i les hores analitzant withdrawal, però sempre encarant-ho tot amb bon humor. A ell li he d'agrair els caps de setmana, Nadal, St. Esteve i reis passats a l'estabulari registrant el two-bottle... ;P Xavi, moltes gràcies per la teva ajuda, estic segura que seràs molt bon investigador i arribaràs lluny. Per cert, ja has après a buscar bolets? ;).

Durant aquests quatre anys he tingut la sort de conèixer a l'**Ignasi Sahún**. D'ençà de la seva mudança, no es pot arribar a imaginar el buit que ens ha deixat al laboratori, un buit que mai ningú podrà substituir, es troba a faltar. Quan feia poc que el coneixia recordo haver-li escrit en un llibre, que en aquest món feia falta més gent com ell. Una persona agraïda, entregada als altres, disposat a posar-te per davant en qualsevol moment i a obrir-te les portes de casa. Avui reafirmo les meves paraules. No em cap descriure tots els moments que hem viscut plegats, entre torneigs de volei, partits de futbol, fideuàs, fi d'anys... Ignasi, espero no perdre mai el contacte, ets un bon amic, un germà gran, que m'agradaria mantenir sempre amb mi.

També he compartit aquests quatre anys amb la **María Martínez de Lagrán**, una persona admirable, una ment investigadora com poques. La María no és conscient del seu potencial, pot estar tant orgullosa de la seva personalitat, capaç de donar-te sempre una resposta, tant a la feina com a la vida del dia a dia. Gràcies María per escoltar-me, corregir-me, animar-me, per compartir les rialles a les danses afrocaribenyes, i el fuet, pistatxos i cerveses entre converses interminables. María, una de les persones que més m'ha ensenyat.. pots arribar on et proposis, ets una dona increïble.

I la **Carla Obradors**, una altra biotecnòloga, això des del primer dia em va fer sentir un carinyo especial. La Carla, és pur esperit de superació, de derrotar qualsevol adversitat i seguir endavant, treballadora com poques. Amb tota la dedicació, ha aconseguit sobrepassar totes les seves metes. Carla, m'has fet despertar tantes rialles, m'has

escoltat, entès, i aconsellat. Hem compartit també hores de danses, i sempre m'enduc un bon record de les nostres trobades. Carla, vals un imperi, espero que tinguis molta sort en tot, t'ho mereixes.

Més endavant va arribar la **Meritxell Pons**, amb qui puc confirmar que a vegades el món és com un mocador. La Meri té un do, és com una formiga, eficaç, va fent mica en mica, i aconsegueix resultats dignes d'admirar, en tots els àmbits. Meri, envejo aquest do, t'espera un futur prometedor, superaràs qualsevol assumpte amb matrícula, sigui el que sigui triomfaràs, n'estic convençuda. M'ha agradat conèixe't, ets una bona companya i una bona amiga, Meri, moltes gràcies per ser-hi sempre.

I el **Davide D'Amico**, un sicilià de cap a peus i un empresari com déu mana. El Davide té una mentalitat clara, sap el què és apostar i arriscar, amb objectius fixes, astut i emprenedor. Amb el Davide he compartit partits de volei, natació a alta mar i inclús córrer pel passeig marítim. Gràcies per ajudar a crear el Pepeta-Arancino, crec que ningú oblidarà mai el que semblava impossible: "Este año no vamos a ver la final, no, este año, la vamos a jugar" (Pepeta-Arancino, 2on classificat i equip més lluitador al torneig de volei platja PRBB 2011). Davide, moltes gràcies per tots aquests moments, aconseguiràs el que vulguis, no en tinc cap dubte.

La **Tiziana Pederzani**, una peça clau al laboratori. Sempre et rep amb un "bon dia", i et pregunta "com estàs". És una persona eficient, treballadora, milers de gràcies per els teus genotipats a últim moment, per controlar que tot vagi bé, tant al laboratori com a fora. Tiziana, espero que siguis molt feliç.

I el **Thomas Gener**, amb qui he discutit hores i hores els resultats de l'electrofisiologia, i també compartit partits de volei a la platja. Thomas, moltes gràcies per la teva paciència, ajuda i comprensió.

Una de les últimes incorporacions, la **Carmen de Agustín Pavón**. La Carmen és un pou de ciència, literatura, cultura, política i de tot. Acompanyada de la seva honradesa i afectació envers als altres, se'ns ha ficat a tots a la butxaca. Carmen, n'hi ha poques com tu.

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Ni del **Nuno Vasconcelos**, tot un vividor, a qui he d'agrair la seva amabilitat i bones paraules. Tampoc vull deixar-me l'**Aida Regi**, una persona amb una força extraordinària, que ens ha ajudat amb el Jtracks i els seus gràfics.

Al laboratori vaig coincidir poc amb el **Gari Azcona**, la **Glòria Arqué** i l'**Àlex Amador**, a tots ells els aprecio molt i desitjo prosperitat en el que facin.

Fora del laboratori, he tingut la sort que la família i els amics m'han acompanyat en tot moment.

La **Marta Téllez**, o millor per mi, sempre serà la Marta de la uni. Marta, admiro la teva força, generositat, valentia i entusiasme. Mai tens un no per paraula, una lluitadora com poques. M'has ensenyat, escoltat, comprès i aconsellat. Ets una gran dona i et vull donar les gràcies per tot i més.

I la **Carol Ricarte**, una persona d'idees fixes, que malgrat la distància, el seu somriure i carinyo sempre està present.

La **Montse Isern** i en **David González**. Heu estat amb mi incondicionalment. Els tres hem viscut junts moments de felicitat i eufòria, junts ens hem ajudat a superar moments de pura desconsol, junts hem cridat al mar. M'alegro d'haver-me creuat amb vosaltres en aquesta vida, gràcies als dos de tot cor.

En Josep Maria Pons, i els seus pares en Joaquim i la Marta, no puc no anomenar-los en aquest moment, per mi tant important. En Josep ha format part de la meva vida durant gairebé nou anys, m'ha acompanyat en la majoria de les etapes que he anat superant i sempre ha estat allà. Gràcies Josep per tot el que hem viscut, ensenyar-me que la vida no

només és treballar sinó que el que compta és tenir il·lusions. Gràcies també als teus pares per acollir-me com una filla.

I per descomptat, les meves germanes, la Cris i la Moni. No puc expressar el que sento per elles amb paraules, elles ho han sigut tot, m'han fet de germanes, mares, amigues, no podrien deixar d'existir a la meva vida. Igual que els meus pares, en Jesús i la Núria, que m'han transmès la força, l'energia, la humilitat i m'han ensenyat a valorar els petits detalls. Tots ells m'han donat suport als bons moments i encara més en moments difícils. En aquest cas, sóc jo la que estic orgullosa, m'omplo de felicitat quan veig la família amb qui he crescut tots aquests anys, gràcies a tots ells, per haver arribat on som.

Finalment, vull donar les gràcies a l'Esteban Javier Rozen. Gràcies, per estar cada dia al meu costat, per fer-me creure que tot es pot arribar a aconseguir, per ensenyar-me a no rendir-me. Gràcies, per haver viscut moments de plena felicitat, valorar el que faig en tot moment i acompanyar-me a qualsevol lloc. Gràcies per tractar-me com una princesa, i per estar disposat a ajudar-me en el que sigui necessari. Escriure aquest tesis ha sigut la meitat de dura sabent que estaves amb mi. Gràcies amor.

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ABSTRACT / RESUM

ABSTRACT

The *CHRNA5/A3/B4* genomic cluster encodes for the alpha5, alpha3 and beta4 subunits of the nicotinic acetylcholine receptors (nAChRs). Human genetic studies have revealed a significant association of variants in this genomic region with nicotine dependence. However, the mechanisms through which overexpression of these three subunits may influence smoking-related behaviours is not understood. To gain insight in the possible mechanisms, we used a BAC transgenic mouse model overexpressing this cluster containing the three genes together with their transcriptional regulatory elements. We found that overexpression of the cluster: i) increases sensitivity to the pharmacological effects of nicotine; ii) modifies particular cognitive domains associated to drug addiction and hippocampal neuronal complexity and synaptic plasticity; and iii) shifts the rewarding and aversive properties of nicotine and the manifestation of nicotine-withdrawal syndrome. Our study suggests that the genomic cluster *CHRNA5/A3/B4* contributes to genetic vulnerability to nicotine addiction and promotes smoking-related behaviours possibly through hippocampal plasticity changes.

RESUM

El cluster genòmic *CHRNA5/A3/B4* codifica per les subunitats alfa5, alfa3 i beta4 dels receptors d'acetilcolina (nAChRs). Estudis de genètica humana han revelat que variants en aquesta regió genòmica estan significativament associats a la dependencia a nicotina. Malauradament, els mecanismes pels quals la sobreexpressió d'aquestes tres subunitats influencia comportaments relacionats amb el consum de tabac no són del tot coneguts. Per tal d'entendre els possibles mecanismes, hem utilitzat un model de ratolí transgènic que sobreexpressa aquest cluster amb els tres gens i les seus elements de regulació transcripcional. Hem trobat que la sobreexpressió del cluster: i) incrementa la sensibilitat als efectes farmacològics de la nicotina; ii) modifica determinats dominis cognitius associats a l'addicció a droges i la complexitat neuronal i plasticitat sinàpica de l'hipocamp; a més a més iii) canvia les propietats de recompensa i aversió de la nicotina i la manifestació del síndrome d'abstinència. El nostre estudi suggereix que el cluster genòmic *CHRNA5/A3/B4* contribueix a la vulnerabilitat genètica a l'adicció a la nicotina i promou comportaments relacionats amb el consum de tabac possiblement a través de canvis de plasticitiat a l'hipocamp.

PRESENTATION

PRESENTATION

The present Doctoral Thesis has been devoted to study genetic vulnerability in nicotine addiction. Specifically, we focus on the cluster *CHRNA5/A3/B4* of human chromosome 15, which human genetic studies have identified as a strong candidate for nicotine dependence and smoking-related behaviours. This cluster codifies for the alpha5, alpha3 and beta4 subunits of the nicotinic acetylcholine receptors (nAChRs), with low and restrictive expression pattern within the central nervous system. Since the majority of these studies address the role of individual subunits, several years ago, we generated a bacterial artificial chromosome (BAC) transgenic mouse model overexpressing the whole human cluster *CHRNA5/A3/B4*. Initially we focused on nicotine and ethanol addiction profiles (Gallego et al, 2011, 2012), but very rapidly we realized that other traits of our transgenic mice, could also contribute to the addictive phenotype. Our previous results (Vinals et al 2012) revealed the involvement of α3/α5/β4 nAChRs subunits in working memory and impulsivity, two behavioural traits directly related to the vulnerability to develop nicotine dependence. This Thesis has been devoted to explore the impact of overexpression of the cluster in reward and learning and memory processes and the differential effects of nicotine in genetically susceptible individuals. The first question to be addressed was if overexpression of nAChRs would increase the sensitivity to the pharmacological effects of nicotine, thus leading more easily to consumption. We then focused on cognitive domains associated to drug addiction and the possible neural plasticity changes in hippocampal neurons derived from nicotine administration. We wanted to determine if genetically susceptible individuals had specific cognitive traits predisposing to drug abuse, and to what extent nicotine was able to modify these phenotypes. Finally, we studied how the differential genetic profiles could influence the rewarding and aversive properties of nicotine and the manifestation of nicotine-withdrawal syndrome.

INDEX

INDEX

INTRODUCTION

1. INTRODUCTION

Tobacco addiction is the second leading cause of preventable mortality in developing countries (Benowitz, 2008), with approximately 5 million deaths per year due to tobacco-related diseases. If current trends in tobacco use persist, by 2020 smoking will become the largest single health problem worldwide, causing an estimated 8.4 million deaths annually. In high-income countries, smoking-related healthcare accounts for between 6 and 15% of all healthcare costs (Tuesta et al., 2011). Hence, it is not surprising that research is devoting huge effort investigating the molecular and cellular causes for tobacco addiction and the possible predisposing/susceptibility factors that could be pharmacologically targeted.

1.1. NICOTINIC ACETYLCHOLINE RECEPTORS (nAChRs)

The plant alkaloid nicotine is a psychoactive tertiary amine responsible for the addictive component of tobacco (Dani and De Biasi, 2001; Mansvelder and McGehee, 2002). Nicotine binds to nicotinic acetylcholine receptors (nAChRs) that belong to the superfamily of ligand-gated ion channels (LGIC), which normally respond to the endogenous cholinergic ligands (i.e. acetylcholine). Acting through nAChRs, nicotine induces and maintains tobacco use (Dani and Heinemann, 1996; Shadel et al., 2000). nAChRs are widely distributed throughout the central nervous system (CNS) and peripheral nervous system (PNS), and numerous studies have also revealed the expression of nAChRs on non-neuronal cells such as lung, glia, keratinocytes, endothelial cells or cells of the digestive and immune systems (Flora et al., 2000; Improgo et al., 2010b).

nAChRs consist of pentameric assemblies of subunits surrounding a central aqueous pore that allows the flux of Na^+, K^+ and Ca^{2+} into the cell (Fig. 1A). Their ability to alter intracellular Ca^{2+}, activating different downstream intracellular pathways suggests nAChRs to play a pivotal role in neuronal signalling (Gotti et al., 2006). Furthermore, their localization is fundamental in determining the functional properties of the receptor. nAChRs can be found postsynaptically, where they influence the probability of reaching the threshold for action potential, contribute to the fast

excitatory transmission, and to activity-dependent gene expression (Fig. 1B) (Albuquerque et al., 2009; Dani and Bertrand, 2007; Hu et al., 2002; Ji et al., 2001). However, nAChRs within the brain are predominantly found presynaptically, where they modulate the release of neurotransmitters such as acetylcholine (ACh), dopamine (DA), norepinephrine and serotonin (5-HT), as well as glutamate and gamma-aminobutyric acid (GABA). Also, they are able to modulate the release of adrenocorticotropic hormone (ACTH), corticotrophin-releasing hormone (CRH) or neuropeptide Y (NPY). Reviewed in (Albuquerque et al., 2009).

Interestingly, nAChRs assemblies are determinant in conferring specific electrophysiological and pharmacological properties to the receptor. A total of 17 distinct genes identified in vertebrates encode nAChRs subunits, thus resulting in a wide array of possible subunit combinations. Many of these combinations have been demonstrated to form functional channels in heterologous expression systems (Le Novere and Changeux, 1995). nAChRs subunits have been classified as α (α2 –α 10) and β (β2-β4), which can assemble to form heteromeric receptors of α and β subunits but also homomeric receptors from exclusively α- subunits[1] (Fig. 1C) (Albuquerque et al., 2009; Dani and Bertrand, 2007; Gotti and Clementi, 2004; Gotti et al., 2009; Jensen et al., 2005).

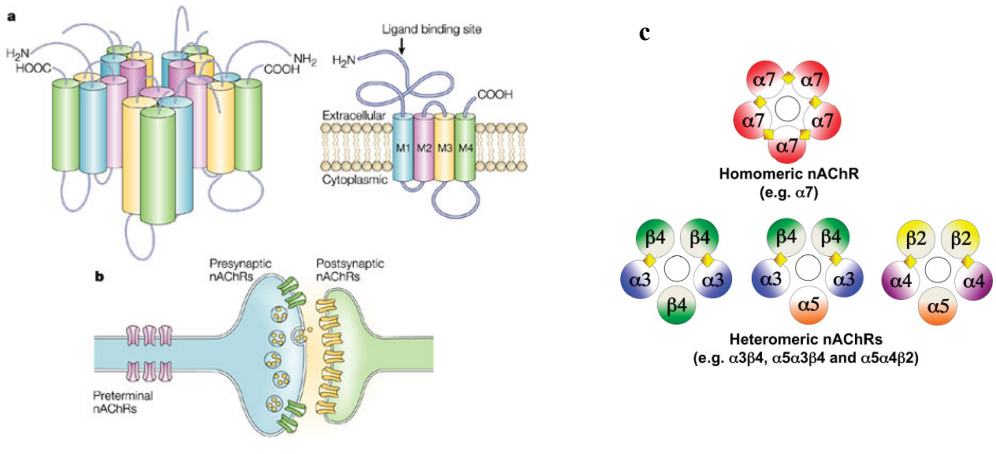


Figure 1. nAChRs structure and localization. (**a**) Schematic representation of pentameric assemblies of nAChRs subunits and individual subunit structure. The amino H2N and carboxy COOH terminals of the protein reside on the extracellular fraction and the subunit has four transmembrane domains labelled M1 – M4. (**b**) nAChRs localize on preterminal, presynaptic and postsynaptic sites. Adapted from *Nature*

[1] Because of the lack of absolutely certainty in determining the specific subunits that are present in native nAChRs, it is common to refer to various receptors as "containing" particular nAChR subunits denoted with an asterisk (i.e. α3β4*)

Reviews Neuroscience 5, 55-65 (Laviolette and van der Kooy, 2004). (**c**) Examples of homomeric and heteromeric nAChR subtypes. Individual receptor subunits are represented as coloured circles. Diamonds located between adjacent receptor subunits represent ligand-binding sites while unfilled circles in the centre of each pentamer represent the pore region. Adapted from *Prog Neurobiol*.; 92(2): 212–226 (Improgo et al., 2010b).

The α subunits have adjacent cysteines in their native structure that are involved in ACh/nicotine binding. Instead, β subunits are important in conferring specific pharmacological properties to the receptor complex (Drago et al., 2003) . In heteromeric receptors the fifth subunit exists as an accessory subunit, which does no directly participate in forming the agonist binding-site but instead, it changes the pharmacological and biophysical properties of the channel, influencing the permeability to calcium and affinity to Ach and nicotine, and the sensitivity to allosteric modulators (Albuquerque et al., 2009; Dani and Bertrand, 2007; Gotti and Clementi, 2004; Gotti et al., 2009; Jensen et al., 2005). Functionally, the nAChRs complex can exist in three conformational states, which are dynamically regulated by exposure to the agonist: closed, open and desensitized (Laviolette and van der Kooy, 2004). Accessory subunits are also important for defining the rate of desensitization of the channel and the susceptibility to be upregulated by nicotine. Prolonged exposure to low concentrations of nicotine, as obtained from tobacco use, produces significant desensitization, which stabilizes the receptor in a closed state that is unresponsive to agonists (Gotti et al., 2009). Receptor desensitization is a main cause underlying nAChRs upregulation, increased receptor function and/or number after chronic exposure to nicotine (see below).

nAChRs consisting of α4β2* or α7* are the most abundant and presumably the most important nAChRs present in the CNS (Fig. 2). Not surprisingly, most studies have focused on these two nAChRs subtypes. The α4β2* is the predominant heteromeric receptor in the brain and binds nicotine with high affinity. The α7* subtype is a homomeric receptor with high permeability to calcium, similar to that observed for N-metyl-D-aspartic acid (NMDA) receptors, and thus, α7* nAChRs have important implications for synaptic plasticity (Broide and Leslie, 1999). In contrast to α4β2*, α7* nAChRs rapidly desensitize after nicotine exposure, but the relative concentration of nicotine needed to achieve such desensitization is higher than for α4β2* nAChRs. Additionally, the α3β4* subtype is predominant in the PNS (autonomic ganglia and

adrenal medulla) although it is also present in the CNS. The α3β4* nAChRs primarily gate Na^+, and desensitize slowly (Albuquerque et al., 2009; Dani and Bertrand, 2007; Gaimarri et al., 2007; Gotti and Clementi, 2004; Gotti et al., 2009; Jensen et al., 2005).

Besides its role in adult, nAChRs show region-specific changes during development (Zhang et al., 1998). Towards birth, nAChRs subunits mRNA levels are highest and then start to decline with the rate depending on the nAChRs subtype but also the brain area (Court et al., 1997; Hellstrom-Lindahl et al., 1998). These findings suggest that nAChRs may play a critical role during development, particularly in morphogenesis and cell survival, and in regulation of gene expression, developmental GABAergic signalling and neurotransmitter release, among others (Dwyer et al., 2008).

Finally, the importance of nAChRs in human diseases of the nervous system has also been addressed. Decline, disruption or altered function of nAChRs lead to serious neurological diseases, such as anxiety, depression, drug addiction, schizophrenia, epilepsy, attention deficit hyperactivity disorder (ADHD), Parkinson's disease or Alzheimer's disease (Dani and Bertrand, 2007; Gotti and Clementi, 2004; Mihailescu and Drucker-Colin, 2000).

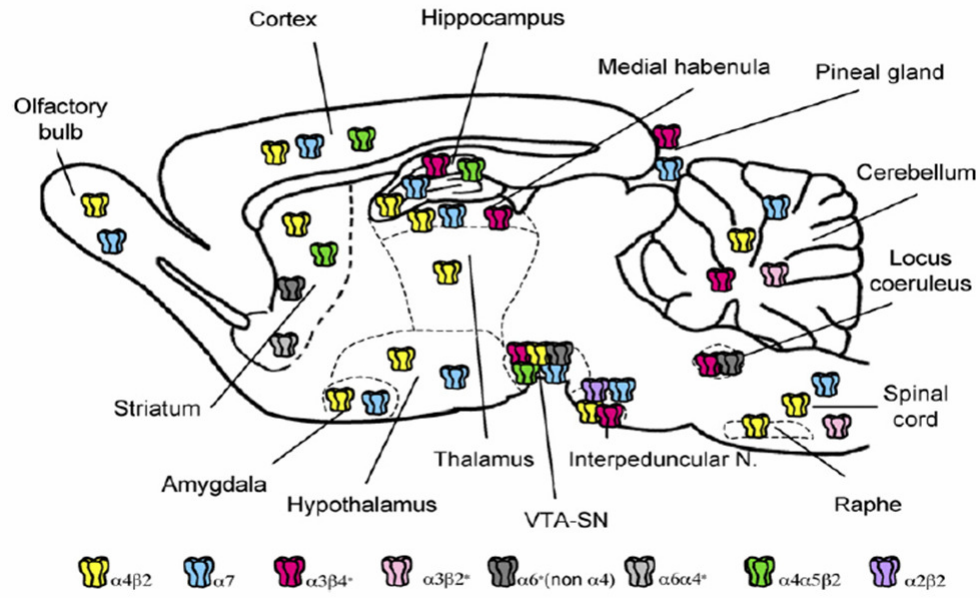


Figure 2. nAChRs distribution in the CNS. Multiple combinations of nAChR subunits exist and are widely distributed throughout the CNS, α4β2 * and α7* nAChRs are the most predominate receptors. Adapted from *Brain Res Rev. 55(1):134-43* (Gaimarri et al., 2007).

1.2.GENETIC VULNERABILITY IN TOBACCO ADDICTION: THE *CHRNA5/CHRNA3/CHRNB4* GENOMIC CLUSTER

Twin studies estimate that the heritability of tobacco addiction is approximately 50% (Lessov et al., 2004), suggesting that genetic factors play a prominent role. Numerous attempts have been made to identify genes underlying tobacco addiction. Not surprisingly, many of these studies have been focused on genes encoding for nicotine receptor subunits as good candidate predictors for tobacco addiction.

In recent years, linkage and candidate-gene analyses, and large-scale genome-wide association studies (GWAS) have identified a genomic cluster on human chromosome 15q24-25, that contains the *CHRNA5, CHRNA3* and *CHRNB4* genes (*CHRNA5/A3/B4*) associated with nicotine dependence, lung cancer and also alcohol dependence (Berrettini and Doyle, 2011; Bierut, 2010; Bierut et al., 2008; Chen et al., 2012; Improgo et al., 2010a; Lubke et al., 2012; Saccone et al., 2010; Saccone et al., 2009; Wassenaar et al., 2011). The cluster codifies for the α5, α3 and β4 subunits of the nAChRs (Boulter et al., 1987; Duga et al., 2001) and is evolutionarily conserved. These three subunits can form functional channels (Ramirez-Latorre et al., 1996), or can also incorporate into other nAChRs, thus conferring specific properties to the resulting channel.

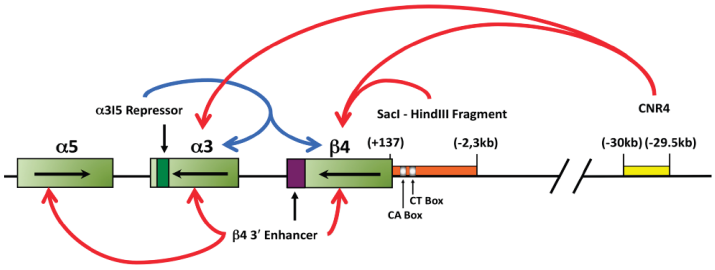


Figure 3. Positive and negative regulation of the clustered nAChR subunit genes. Coding regions of the clustered subunits are represented as light green boxes with arrows that indicate the direction of transcription. Four transcriptional regulatory elements are depicted in this figure: A3I5 (green box), the β4 enhancer (purple), the SacI – HindIII fragment of the β4 promoter region (orange box), and the distal CNR4 regulatory region (yellow box). Red arrows denote positive regulatory effects whereas blue arrows indicate negative transcriptional regulation. Adapted from *Prog Neurobiol.*; 92(2): 212–226 (Improgo et al., 2010b).

These genetic studies have been confirmed in multiple human populations and when evaluating several clinical parameters of nicotine addiction (age of onset, heaviness of smoking, pleasurable effects of the first cigarette exposure, successful quitting etc.) (Berrettini et al., 2008; Freathy et al., 2009; Kapoor et al., 2012; Schlaepfer et al., 2008; Stevens et al., 2008; Weiss et al., 2008; Zhang et al., 2010). One of the single nucleotide polymorphisms (SNPs) associated with nicotine dependence is a non-synonymous SNP, rs16969968, located on exon 5 of the α5 gene that changes an aspartic acid residue into asparagine at position 398 (D398N) and appears to influence the biophysical properties of α5* nAChRs (Bierut et al., 2008). Individuals with one copy of the risk variant have a 1.3-fold increased risk for nicotine dependence while individuals with two copies have almost a 2-fold increase in risk (Improgo et al., 2010a).

However, most of the SNPs were located on non-coding regions, suggesting that a deregulated expression of these nAChRs subunits may lead to increased dependence (Falvella et al., 2010; Schlaepfer et al., 2008; Wang et al., 2009; Xu et al., 2006). This is of particular interest since the three genes have their own promoter region but they share same regulatory elements (Francis and Deneris, 2002; McDonough and Deneris, 1997; Xu et al., 2006) (Fig. 3). In fact, their clustering may reflect coordinate regulation, as supported by the fact that the genes are co-expressed in many cell types, and the transcriptional activities of the promoter regions of the three genes are regulated by the same transcription factors (Improgo et al., 2010b).

As mentioned, α3β4* nAChRs predominate in the PNS, together with the α5 subunit. The prominent role of these subunits arises from knockout (KO) mice. Mice that do not express the α3 subunit usually die within a week after birth due to multi-organ dysfunction (Xu et al., 1999a). In contrast, α5 and β4 KO mice are both viable and lack any gross abnormalities (Wang et al., 2003; Wang et al., 2002; Xu et al., 1999a; Xu et al., 1999b), only showing impaired ganglionic transmission. Within the CNS, the three subunits show a restricted expression pattern. The α3 subunit is expressed in the brainstem, cerebellum, spinal cord, substantia nigra (SN), medial habenula (MHb), pineal gland, hippocampus, cortex, thalamus, ventral tegmental area (VTA) and interpeduncular nucleus (IPN) (Gahring et al., 2004; Guan et al., 2002; Hellstrom-Lindahl et al., 1998). The β4 subunit is expressed in the olfactory bulb, pineal gland, MHb and IPN, with lower expression in other thalamic nuclei, cerebral

cortex, hippocampus, spinal cord, cerebellum and midbrain (Azam et al., 2002; Gahring et al., 2004; Hellstrom-Lindahl et al., 1998; Perry et al., 2002). Finally, the α5 subunit is expressed primarily in the cerebellum and thalamus but is also detected in the cortex, hippocampus, brainstem, spinal cord, MHb, IPN and other midbrain nuclei (Flora et al., 2000; Gahring and Rogers, 2010; Grady et al., 2009; Hellstrom-Lindahl et al., 1998; Wada et al., 1989). Noticeably, the three subunits are expressed in the habenula-interpeduncular nucleus and the hippocampus, two brain regions involved in tobacco addiction.

Interestingly, the α5 subunit is not able to form the ligand-binding site, and instead functions as an accessory subunit. Incorporation of α5 subunit into nAChRs completely changes the pharmacological and functional properties of the receptor (Wang et al., 1996). It increases the sensitivity of α3β2 nAChRs to nicotine and ACh, but does not produce substantial effect in α3β4 nAChRs. Additionally, co-assembly of α5 subunit with α3β2 or α3β4 nAChRs increases the calcium permeability and the rate of desensitization of the resulting receptor (Gerzanich et al., 1998).

Currently, the majority of studies that address the role of the α5, α3 and β4 nAChRs subunits have either knocked down or overexpressed individual subunits, but none of them have explored the entire cluster. Unfortunately, pharmacological tools that target specifically these subunits are readily questioned.

1.3. THE NEUROCIRCUITRY OF TOBACCO ADDICTION

1.3.1. Reward and initiation of addiction

Nicotine is addictive even in the absence of tobacco. It is self-administered by humans, non-human primates, dogs, rats, and mice (Corrigall et al., 1999; Picciotto, 1998). It acts directly on neural circuitries that normally reinforce behaviours leading to rewarding goals (De Biasi and Dani, 2012) and subsequently stimulating repetition of those learned behaviours (Koob and Volkow, 2010; Schultz, 2010). The mesocorticolimbic dopaminergic (DA) system (Fig. 4) plays an important role in processing these reward-related stimuli and in the acquisition of addictive-related behaviours, such as self-administration (Dani and De Biasi, 2001; Dani et al., 2012; De

Biasi and Dani, 2012; Laviolette and van der Kooy, 2004; Olds and Milner, 1954). The origin of the mesocorticolimbic DA system is the ventral tegmental area (VTA). The VTA receives afferent innervation from several regions, the most important are the pedunculopontine tegmentum (PPTg), the laterodorsal tegmentum (LDTg) and the prefrontal cortex (PFC), which modulate the firing of DAergic neurons and processing reward-based information (Chen et al., 2011; Omelchenko and Sesack, 2005) (Fig 4). VTA fibers target regions involved in reinforcement (nucleus accumbens, NAc), learning and declarative memory (hippocampus), emotional memory (amygdala), and habit forming (dorsal striatum), as well as executive functions and working memory (PFC and orbitofrontal cortex) (Kauer and Malenka, 2007; Laviolette, 2007).

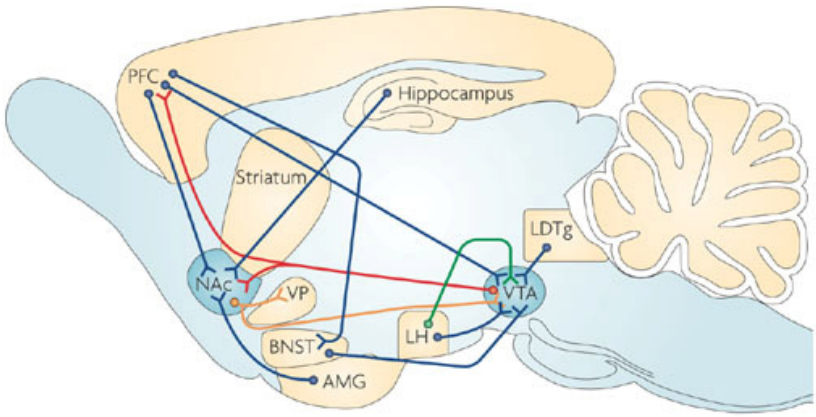


Figure 4. Simplified schematic of the circuitry of the mesolimbic dopamine system in rodent brain. Blue = glutamatergic projections, red = dopaminergic projections; orange = GABAergic projections; green = orexinergic projections. AMG, amygdala; BNST, bed nucleus of the stria terminalis; LDTg, laterodorsal tegemental nucleus; LH, lateral hypothalamus; PFC, prefrontal cortex; VTA, ventral tegmental area; VP, ventral pallidum. Adapted from *Nature Reviews Neuroscience 8, 844-858* (Kauer and Malenka, 2007).

Nicotine rewarding properties rely on its ability to increase the burst firing of DAergic neurons (De Biasi and Dani, 2012; Mansvelder and McGehee, 2002; Rice and Cragg, 2004; Schilstrom et al., 1998; Zhang et al., 2009) thereby elevating DA levels in the NAc. Increases in NAc DA are crucial in processing reward-related information and thus, are important for the process of drug addiction (Grace, 2000; Schultz, 2010). Importantly, the DA system broadly influences neural processing that underlies reward-based memory and behaviour. Indeed, addiction and learning involve common neural areas and molecular substrates, and addiction-related changes in processes underlying plasticity may contribute to addiction (Gould, 2006; Kauer and Malenka, 2007; Kelley,

2004). During the last years several studies have addressed the role of DA signalling in reward learning, and concluded that DA does not signal reward itself but reward expectation (Matsumoto and Hikosaka, 2007; Matsumoto and Hikosaka, 2009a; Matsumoto and Hikosaka, 2009b). These findings suggest DA to be a learned signal, and further strength the hypothesis that addiction represents a learned behaviour.

Behavioural and functional studies have demonstrated that α4 and β2 subunits are sufficient for triggering nicotine-mediated DA elevation in the NAc and acquisition of nicotine self-administration (Marubio et al., 2003; Pons et al., 2008), though other nAChRs are also relevant. Indeed direct nicotine infusion into the NAc facilitates the release of DA through activation of presynaptic α4α6β2β3 and α6β2β3 nAChRs (Pons et al., 2008; Yang et al., 2011). In the mesocorticolimbic DA system, (α4β2)α5 have been demonstrated to regulate cortical GABA as well as striatal DA (Grady et al., 2010) release. It seems likely that the α5 subunit is effectively implicated in regulating the direct rewarding effects of nicotine. Although the α3 is also expressed within the VTA it seems to play less important role than the α5 subunit.

1.3.2. Aversion and control of nicotine intake

When access to a range of nicotine doses is provided, nicotine is self-administered according to an inverted 'U' shaped dose-response (D–R) curve (Picciotto, 2003). The ascending limb of the D–R curve is hypothesized to reflect the increasing reinforcing effects of nicotine, motivating a greater response, while the descending limb represents increasing aversive drug effects or rapid development of drug satiation, which act to limit responding (Tuesta et al., 2011). The strong PNS activation at high nicotine doses, along with activation of heterogeneous population of nAChR subtypes in specific CNS neuronal circuits mediates aversive effects of nicotine (De Biasi and Dani, 2012; Laviolette and van der Kooy, 2001). This shape of responding suggests that smokers titrate their nicotine intake to experience the rewarding and avoid the aversive actions of the drug (Benowitz, 2010; Benowitz and Jacob, 2001; Dani and Heinemann, 1996; Robinson et al., 1996).

Aversive sensory information is processed by the habenular complex, an epithalamic structure that receives substantial inputs from multiple parts of the limbic system (i.e. PFC, hippocampus, NAc). Specific components of the habenula pathway

are involved in the physiology of reward and subserve a variety of behaviours such as nociception, stress, sleep, eating and cognition (Baldwin et al., 2011; De Biasi and Salas, 2008; Klemm, 2004; Sanders et al., 2009). Structurally, the habenula is divided into two functionally distinct core regions, the lateral (LHb) and the medial habenula (MHb). Human and monkey studies (Matsumoto and Hikosaka, 2007; Matsumoto and Hikosaka, 2009a; Shepard et al., 2006) indicate that the LHb is activated by negative reward signals or by absence of positive reward, LHb neurons increase their firing in the absence of predicted reward and decrease their firing upon delivery of reward, opposite to firing of DAergic neurons linked to positive reward value (Baldwin et al., 2011; De Biasi and Dani, 2012; Hikosaka et al., 2008; Matsumoto and Hikosaka, 2007; Matsumoto and Hikosaka, 2009a). LHb thus modulates VTA firing participating in reward-learning processes. Hence, drugs of abuse have been proposed to produce improper coordinated firing and activity between DAergic and habenular neurons thus facilitating maladaptive reward learning and maintaining substance consumption.

On the other hand, MHb primarily receives inputs from the limbic system and sends projections to the IPN via the fasciculus retroflexus, which in turn projects to the VTA (Klemm, 2004). MHb neurons express α3, α4, α5, β2 and β4 nAChRs subunits being α3β4* nAChRs the most abundant receptors in the mouse habenula (Grady et al., 2009; Scholze et al., 2012). Several lines of evidence point the MHb as a critical region in controlling the aversive properties of nicotine that limit intake of the drug (Fowler et al., 2011; Glick et al., 2002). Interestingly, habenular α5 and β4 nAChRs subunits control nicotine intake (Fowler et al., 2011; Frahm et al., 2011). Deletion of MHb α5 subunit using a lentiviral vector increased nicotine self-administration at high doses of the drug (Fowler et al., 2011), similar to α5 KO mice (Fowler et al., 2011; Jackson et al., 2010). Additionally, a β4 overexpressing transgenic mouse model (Tabac mice) show reduced nicotine intake and interestingly, lentiviral mediated expression of the mutant α5 subunit (D398N) reverses this phenotype (Frahm et al., 2011). Thus, aversion to nicotine may be regulated by the balanced activity of MHb α5 and β4 subunits. Neurons in the MHb are cholinergic (Grady et al., 2009), glutamatergic (Girod et al., 2000), or both cholinergic and glutamatergic (Ren et al., 2011). The primary output of the MHb is the IPN, and presynaptic α5* nAChRs on MHb afferents to the IPN are though to regulate glutamate, but not ACh release in the IPN (Girod et al., 2000; Hussain et al., 2008), which is mediated by β4* nAChRs (Grady et al., 2009).

The exact mechanism through which nAChRs subunits control nicotine intake is not fully understood, even less is known about the MHb and IPN function in regulation of drug self-administration, two regions that are critically involved in withdrawal symptoms that appear upon cessation of nicotine intake (Salas et al., 2009).

The balance between the rewarding and the aversive properties of nicotine depends on the activation state, connectivity and sensitivity of the neuronal pathways that are the site of nicotine action, and determines variations in behavioural responses among individuals (Picciotto, 2003). Upon initial exposure to nicotine, individuals who have higher sensitivity to its rewarding effects and/or individuals experiencing fewer aversive effects of smoking are more likely to subsequent repetition of self-administration (Janet Audrain-McGovern, 2009). Thus, individual differences in nAChRs subunits expression and/or assemblies may determine initial sensitivity to nicotine and therefore vulnerability to smoking initiation and maintenance.

Accumulating evidence suggests that the effects of acute nicotine may provide clues about mechanisms of tobacco addiction, given that the effects of initial exposure to nicotine are a good predictor of later tobacco addiction (Robinson et al., 1996). The α5, α3 and β4 subunits of the nAChRs have been involved in the acute effects of nicotine. Previous reports demonstrated that heterozygous mice for the α3 subunit and KO mice for the α5 and β4 nAChRs subunits are highly resistant to the hypolocomotor effects of nicotine and to nicotine-induced seizures (Kedmi et al., 2004; Salas et al., 2004a; Salas et al., 2003). In rodents, sensitivity to nicotine-induced seizures shows a positive correlation with nicotine intake when nicotine is presented at high doses (Robinson et al., 1996). Furthermore, sensitivity to nicotine-induced seizures and nicotine intake are both highly influenced by genetic factors and correlate with nAChRs levels in the hippocampus (Miner and Collins, 1989; Miner et al., 1985) and the number of nAChRs binding sites in the hippocampus and cortex (Li et al., 2005). Interestingly, the α5 and β4 subunits mediate both aversion to nicotine and sensitivity to seizures. Understanding the conveying pathways on both nicotine-induced seizures and nicotine intake may elucidate cellular and molecular substrates targeted for new therapeutic strategies.

1.3.3. Neural adaptations upon chronic exposure to nicotine

The transition from occasional drug use to addiction involves neuroplasticity in specific brain regions, more importantly, in those related to reward-learning processing (De Biasi and Dani, 2012; Koob and Le Moal, 2005). Chronic exposure to nicotine leads to nicotine dependence that includes specific biochemical alterations, which would promote the physical need for more nicotine to compensate the disarrangement caused by the drug (Ortells and Arias, 2010).

a. nAChRs desensitization and upregulation

Chronic nicotine exposure causes receptor desensitization, leading to a reversible reduction in nAChRs response (Giniatullin et al., 2005). Multiple states of desensitization and/or inactivation exist (Ibanez-Tallon et al., 2002) so that desensitization by an agonist depends on the concentration and length of exposure, with long exposures resulting in deeper states of desensitization that recover more slowly (Quick and Lester, 2002).

Chronic nicotine exposure also elicits increases in high-affinity nAChRs binding sites (termed upregulation) in the brains of mice (Marks et al., 1983; Marks et al., 2004), rats (Perry et al., 2007; Schwartz and Kellar, 1983), and humans (Breese et al., 1997; Perry et al., 1999). nAChRs upregulation lasts beyond cessation of nicotine treatment (Gould et al., 2012) and elevated nAChRs levels correlate with urge to smoke (Mamede et al., 2007). nAChRs upregulation has been proposed to reflect a homeostatic response to compensate the desensitization of receptors due to chronic nicotine exposure (Dani and Heinemann, 1996; Picciotto et al., 2008). Receptor upregulation and receptor desensitization are commonly viewed as opposite, homeostatic mechanisms and are associated with development of nicotine dependence.

Although chronic nicotine exposure generally increases the density of α4β2*-nAChR sites in rodent brain, the extent of the increase varies among brain regions. Moreover, within individual brain regions there is selective upregulation among cell types (Marks et al., 2011; Nashmi and Lester, 2007; Shadel et al., 2000). Interestingly, nAChRs containing the α5 or β4 subunit are highly resistant to nicotine-induced upregulation (Mao et al., 2008; Perry et al., 2007; Sallette et al., 2004; Wang et al.,

1998), which may suggest that altered expression of these subunits may have consequences on nicotine dependence.

b. Tolerance and withdrawal syndrome

Tolerance and withdrawal are the physical manifestation of nicotine dependence (Kauer and Malenka, 2007). Pharmacologically, tolerance can be defined as a shift to the right of the dose-response curve, reflecting the need of a larger dose to achieve the same physical and physiological effects of the drug (Shadel et al., 2000) and nAChRs desensitization might represent a cellular basis of nicotine tolerance (Laviolette and van der Kooy, 2004). Cessation of chronic nicotine administration leads to the expression of withdrawal syndrome. As occurs with other drugs, nicotine dependence has been proposed to be the consequence of avoidance of the aversive effects that appear upon cessation, through negative reinforcement mechanisms (Hughes, 2007). Nicotine withdrawal syndrome involves mood-oriented (affective) and physical (somatic) signs (De Biasi and Salas, 2008; Hughes and Hatsukami, 1986), and reflects the homeostatic neuroadaptations induced by chronic exposure to nicotine (De Biasi and Dani, 2012; Hughes, 2007; Kauer and Malenka, 2007; Koob and Simon, 2009; Koob and Volkow, 2010). Withdrawal may be the cause that a high percentage of smokers attempt to quit but very few succeed (Dani et al., 2012). Thus, the design of effective anti-tobacco pharmacotherapies is aimed at counterbalancing the neuroadaptations produced by chronic nicotine exposure.

Withdrawal symptoms can be assessed in animals chronically exposed to nicotine by sudden discontinuation of nicotine treatment or by administration of nAChRs antagonists (De Biasi and Salas, 2008). They are associated with a decrease in extracellular DA levels in the mesocorticolimbic DA system (Duchemin et al., 2009; Melis et al., 2005; Rahman et al., 2004) and hyperactivity of the habenula (Baldwin et al., 2011). The nicotinic acetylcholine receptor subunits expressed in the MHb are necessary to observe withdrawal symptoms in mice, and blocking nicotinic activity in this region only is sufficient to precipitate withdrawal in dependent mice. Mecamylamine, an antagonist of the nAChRs which has a slightly higher affinity for $\alpha 3\beta 4^*$ nAChRs (Papke et al., 2001) is the best antagonist to precipitate nicotine withdrawal (Damaj et al., 2003). Interestingly, somatic signs of nicotine withdrawal can

be precipitated when mecamylamine is directly infused into the MHb or IPN, but not other brain regions such as VTA, cortex or hippocampus, suggesting the involvement of the MHb/IPN axis in somatic signs of withdrawal associated to chronic nicotine cessation (Salas et al., 2009). A major unanswered question is the mechanism by which the habenula and its connecting structures change activity chronically after repeated nicotine use. KO mice for the α5 and β4 subunits show decreased somatic signs of withdrawal (Jackson et al., 2008; Salas et al., 2007; Salas et al., 2004b; Stoker et al., 2011) but retain affective withdrawal signs. However, β4 KO mice present delayed onset of the anhedonic aspects of spontaneous nicotine withdrawal (Stoker et al., 2011). Other nAChRs, such as those containing the β2 subunit, are more involved in affective signs of withdrawal (Jackson et al., 2008). Some nAChRs have been shown to participate in somatic and affective sins of withdrawal. Further investigation is needed for successful anti-tobacco therapies development.

1.4. THE ROLE OF HIPPOCAMPAL COGNITION IN TOBACCO ADDICTION

Several observations have provided insights into the possible involvement of a deregulated hippocampus in human tobacco addiction (Gould, 2006; Gould et al., 2012). The hippocampus is a brain structure that plays a key role in associative memory networks, the encoding and consolidation of novel information and in the learning of relational information between environmental stimuli (de Hoz et al., 2003). One factor that could contribute to the high addiction rate associated with nicotine is its ability to alter hippocampus-dependent learning and synaptic plasticity (Kenney and Gould, 2008a). The hippocampus gates the flow of novelty and reward-related information to the NAc (Grace, 2000), and smoking-related cues change hippocampal activity in smokers (Franklin et al., 2007). Interestingly, changes in the function of nAChRs in the hippocampus strengthen synaptic connections in efferent areas involved in both reward and learning, such as PFC, striatum or amygdala (Gould, 2006; Placzek et al., 2009). The contribution of the hippocampus in tobacco addiction can be analyzed from two different points of view. On one hand, the hippocampus plays an important role in contextual associations that lead to drug craving. During a smoking cessation attempt,

most relapse episodes occur following exposure to smoking cues (Shiffman et al., 1996). Smoking cues may trigger smoking-related associations leading to the mental rehearsal of past smoking experiences, thus leading to relapse. On the other hand, nicotine is a powerful cognitive enhancer, particularly, hippocampal-dependent learning, and this effect has been proposed to contribute in initiation and maintenance of tobacco consumption.

1.4.1. Contextual associations and craving

With repeated nicotine exposure specific moods, situations, or environmental factors become associated to the reinforcing properties of the drug. Numerous studies have demonstrated that the hippocampus is involved in developing context-drug associations (Kenney and Gould, 2008a). Human neuroimaging studies show that exposure to conditioned stimuli activate the PFC, the basolateral amygdala and hippocampus, and thus all are critical in drug- and cue-induced craving in humans (Franklin et al., 2007; Volkow, 2004). The presentation of smoking cues together with the manifestation of withdrawal symptoms readily contributes to craving and further cigarette smoking, and maintains the nicotine addiction circuitry (Benowitz, 2010)(Fig. 5). Such attentional shifts to drug cue-induced memories and internal states may contribute to increased interference by smoking cues, possibly increasing relapse vulnerability. The correlations between attentional bias and hippocampal and parahippocampal activations suggest that smoking-related memories may be triggered by cues. Smokers with elevated attentional biases to smoking-related stimuli may more readily shift attention away from other external stimuli and toward smoking stimuli-induced internal states and emotional memories.

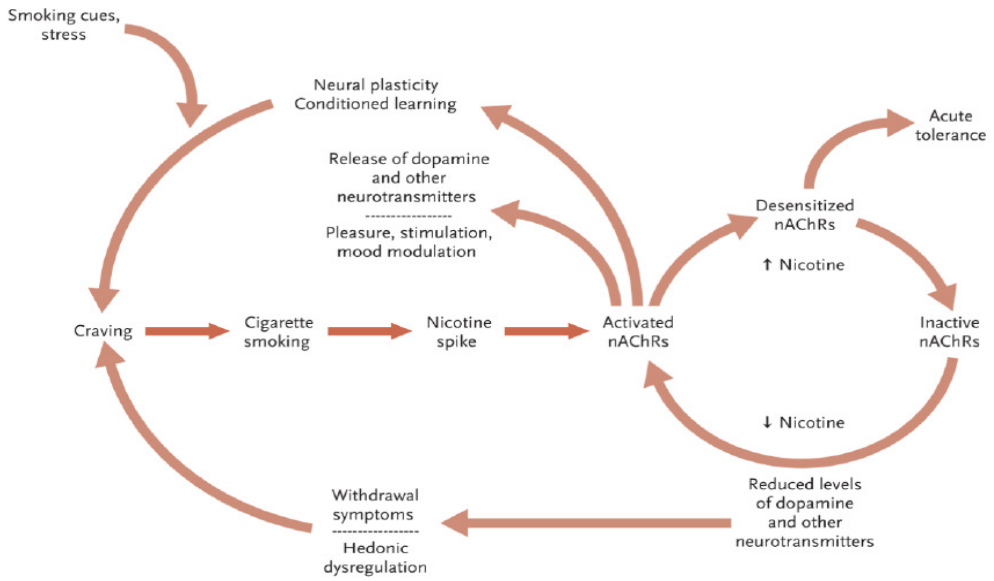


Figure 5. Molecular and behavioural aspects of nicotine addiction. Craving induced by smoking cues, stressors, or the desire to relieve withdrawal symptoms triggers the act of smoking a cigarette, which delivers a spike of nicotine to the brain. Nicotinic cholinergic receptors (nAChRs) are activated, resulting in the release of DA and other neurotransmitters, which in turn cause pleasure, stimulation, and mood modulation. Receptor activation also results in the development of new neural circuits (neural plasticity) and, in association with environmental cues, behavioural conditioning. After being activated by nicotine, nAChRs ultimately become desensitized, which results in short-term tolerance of nicotine and reduced satisfaction from smoking. In the time between smoking cigarettes, or after quitting tobacco use, brain nicotine levels decline, which leads to reduced levels of DA and other neurotransmitters and to withdrawal symptoms, including craving. In the absence of nicotine, nAChRs regain their sensitivity to nicotine and become reactivated in response to a new dose. Adapted from *N Engl J Med. 362(24): 2295–2303* (Benowitz, 2010).

1.4.2. The cognitive-enhancing effects of nicotine

The strong involvement of cholinergic signalling and nAChRs in cognitive function has been recognized for several decades (Drever et al., 2011; Levin, 1992; Levin et al., 2006; Poorthuis et al., 2009). Nicotine and nicotinic agonists improve certain types of memory-related behaviour both in humans and in a variety of animal models (Levin et al., 2001; Rezvani and Levin, 2001; Stolerman et al., 1995). However, nicotine and nAChRs function seem to affect only specific forms of memory (Waters and Sutton, 2000) suggesting a role as modulators (Placzek et al., 2009). When investigating the cognitive-enhancing effects of nicotine in human population some discrepancies among laboratories exist, which may reflect differences in experimental conditions (timing, dose or route of administration) or variations in the level of basal

cholinergic function between individuals. It has been proposed that if the cholinergic functioning of an individual is normal, manipulation of nicotinic function may not produce observable cognitive improvements. Instead, if the subject presents delayed cholinergic functioning, then nicotinic activity can serve to improve or normalize performance (Placzek et al., 2009). This idea is reinforced by the fact that positive effects of nicotine on cognitive function are stronger in specific subpopulations, such as individuals with attention deficit hyperactivity disorder (ADHD) or schizophrenia (Gehricke et al., 2007; Kollins et al., 2005) in which tobacco use may reflect attempts at self-medication. In fact, tobacco use is most highly prevalent and intense in these populations, being 40% observed in ADHD and the 90% in schizophrenia in comparison to general population (26%) (Mihailescu and Drucker-Colin, 2000) .

Importantly, nicotine withdrawal results in cognitive impairments specifically related to hippocampus-dependent learning (Gould et al., 2012; Kenney et al., 2012b; Raybuck and Gould, 2009). Moreover, nAChRs upregulation in the hippocampus contributes to cognitive deficits observed during withdrawal (Gould et al., 2012) and abstinence-associated changes in cognition might predict relapse for smokers (Patterson et al., 2010; Rukstalis et al., 2005).

1.4.3. Molecular and cellular substrates correlates of learning, memory, and addiction

Numerous studies indicate that both learning and addiction share similar molecular and cellular substrates (Gould, 2006). In the case of nicotine, this is of particular relevance since the same substrates are required for its cognitive enhancing and addictive properties.

Activation of nAChRs initiates a cascade of cellular signals, beginning with the influx of Na^+ but also Ca^{2+} cations, which can produce sufficient depolarization to activate voltage-gated calcium channels (VGCC). If nAChRs stimulation is sufficient, prolonged elevations of intracellular Ca^{2+} can occur by the release of free Ca^{2+} from internal stores that will activate second-messenger systems, such as calcium-calmodulin-dependent kinase (CaMKII) and mitogen-activated protein kinase (MAPK). In nAChRs that localize presynaptically, activation of CaMKII facilitate the release of different neurotransmitters (Wonnacott, 1997; Wonnacott et al., 1990). Whereas in

postsynaptic nAChRs, activation of CaMKII leads to initiation of intracellular signalling cascades with subsequent long-lasting phosphorylation of cAMP response element-binding protein (CREB) in the nucleus, and increase gene expression (Albuquerque et al., 1997; Buccafusco et al., 2005; Li et al., 2012)(Fig. 6).

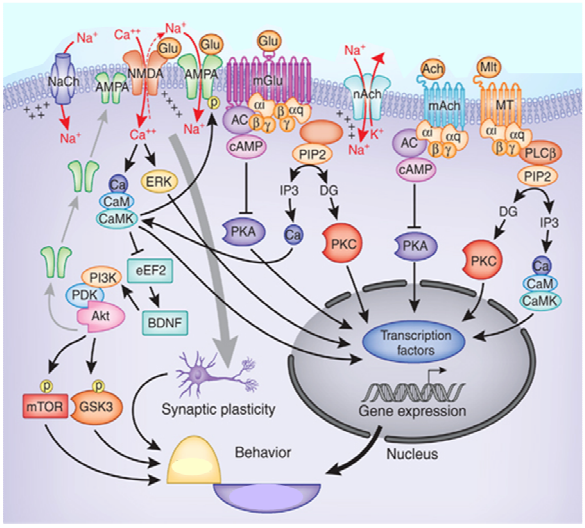


Figure 6. Regulation of intracellular signalling by glutamate and acetylcholine. Activation of ionotropic glutamate receptors or nAChRs increases intracellular sodium and calcium to regulate downstream protein kinases such as calcium calmodulin dependent kinase (CaMK) and extracellular regulated kinase (ERK), and the subsequent activation of transcription factors in the nucleus, and increase expression of the genes involved in synaptic plasticity. Adapted from *Neuropsychopharmacology 37, 77-101* (Li et al., 2012).

Nicotine administration activating nAChRs located at presynaptic terminals and facilitating the release of excitatory neurotransmitter, or activating nAChRs located at postsynaptic sites is able to induce synaptic plasticity at excitatory synapses in the VTA, hippocampus and PFC (Dani and De Biasi, 2001; Placzek et al., 2009). Nicotine can shift short-term potentiation (STP) into long-term potentiation (LTP), but also induce long-term depression (LTD) (Drever et al., 2011; Kauer and Malenka, 2007; Mao et al., 2011). All these forms of synaptic plasticity are induced in the hippocampus after nicotine exposure and have been associated with memory. The direction of synaptic change is strictly dependent on the localization of nAChRs and on the timing of their activation (Ge and Dani, 2005; Ji et al., 2001; Mansvelder and McGehee, 2002; Nakauchi and Sumikawa, 2012). Moreover, NMDA mechanisms, which are critical for synaptic plasticity and learning processes, account for the actions of nicotine in many

brain regions (Aramakis and Metherate, 1998; Mansvelder and McGehee, 2000; Mansvelder and McGehee, 2002).

Evidence indicates that these effects of nicotine on synaptic plasticity facilitate cue-context associations with smoking behaviour that use to precipitate drug cravings (Dalley et al., 2009; Dani and Bertrand, 2007; Shiffman et al., 1996), required for the subsequent development of addiction (Tang and Dani, 2009), and learning of goal-directed behaviours.

Furthermore, exposure to nicotine not only has consequences at the level of synaptic plasticity but also induces structural plasticity. Drug-induced plasticity has been shown to involve altered dendritic branching or arborisation as well as changes in the density or morphology of dendritic spines (Dietz et al., 2009). Dendritic spines cover various shapes and sizes, thought to reflect differences in functional properties (Fig. 7A) (Bourne and Harris, 2008; Hotulainen and Hoogenraad, 2010; Nimchinsky et al., 2002) that correlate with learning and memory processes. Dendritic spines are highly dynamic, because they are actin enriched and polymerization and rearrangement of filamentous actin is critical for the regulation of spine growth and morphological plasticity (Fig. 7B). It has been shown that the size of the spine head is proportional to the size of the postsynaptic receptor density and presynaptic active zone, suggesting that larger spines are indicative of stronger connections and these changes in the structure of dendritic spines can result in an alteration of synaptic function (Bourne and Harris, 2008; Hotulainen and Hoogenraad, 2010). In fact, several studies have demonstrated that nicotine changes the length of dendrites and spine density of excitatory synapses in specific brain regions involved in addiction (Brown and Kolb, 2001; Robinson and Kolb, 2004) (Fig. 7C). Moreover, chronic nicotine exposure leads to remodelling of synapses, producing changes in scaffolding proteins, such as postsynaptic density (PSD) PSD95 and shank (Hwang and Li, 2006). These components of the postsynaptic density are involved with the maturation of dendritic spines, and may play a role in plasticity associated with exposure to drugs of abuse (Dietz et al., 2009).

The overall neuroadaptations contribute to the mechanisms that maintain nicotine consumption, including (Tang and Dani, 2009) facilitation of cue-context associations with smoking behaviour that precipitate drug cravings (Dani and Bertrand, 2007; Shiffman et al., 1996) but also enhancement of cognitive function.

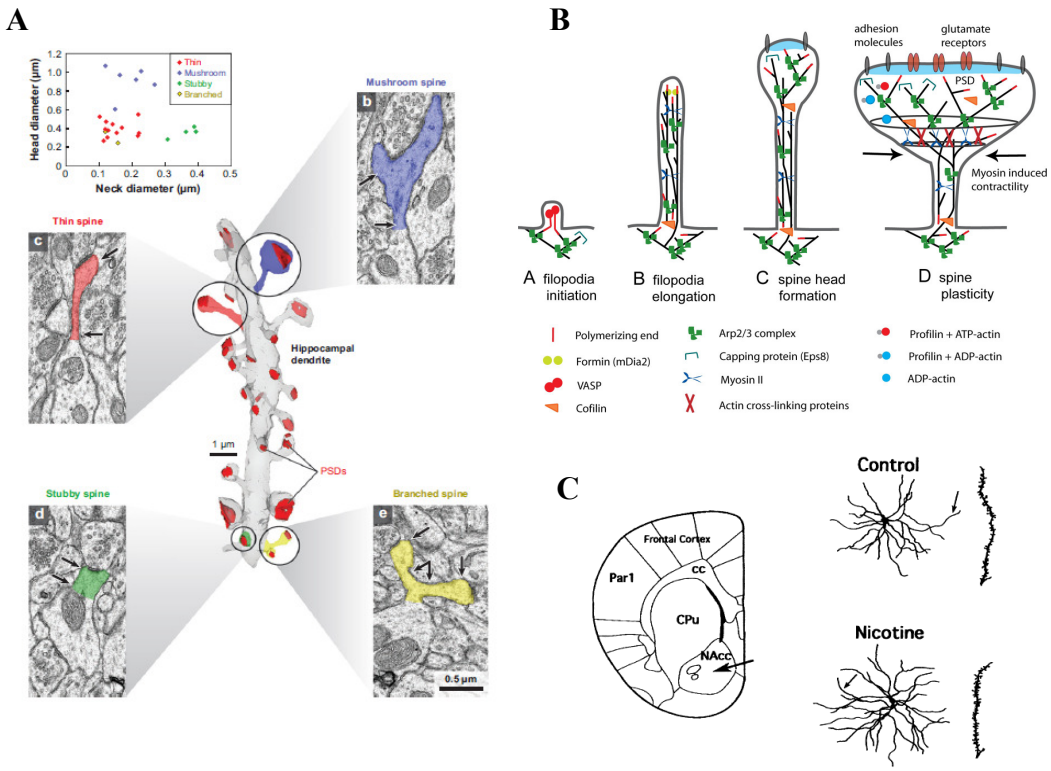


Figure 7. Structural plasticity. (**A**) Dendritic spines cover various shapes and sizes, which can be divided into general categories such as stubby (type-I), mushroom-like (type-II), and thin (type-III), that are thought to reflect differences in functional properties. Branched spines are also represented. Adapted from *Annual Review of Neuroscience vol. 31: 47-67* (Bourne and Harris, 2008). (**B**) Image illustrates the molecules involved in polymerization and rearrangement of filamentous actin to shape a dendritic spine. Adapted from *JCB vol. 189 no. 4 619-629* (Hotulainen and Hoogenraad, 2010). (**C**) Nicotine exposure increases the dendritic branching and the number of dendritic spines in neurons from the NAc. Adapted from *Journal of Communication Disorders Vol 44, 5: 503-514* (Brown and Kolb, 2001).

1.4.4. Hippocampal nAChRs

The hippocampus receives rich cholinergic innervations, mainly from the medial septum-diagonal band complex (Dani and Bertrand, 2007). nAChRs are highly expressed throughout the hippocampus (Albuquerque et al., 1997; Wada et al., 1990; Wada et al., 1989), being the majority of nAChRs subunits located on interneuron population, but also on pyramidal cells (Albuquerque et al., 1997; Alkondon and Albuquerque, 2001; Alkondon et al., 1996; Sudweeks and Yakel, 2000). Hippocampal nAChRs have been identified as being expressed at presynaptic and preterminal sites, as

well as being found at the soma and dendritic sites of both excitatory and inhibitory neurons (Marks et al., 1996; Wada et al., 1989).

Activation of presynaptic hippocampal nAChRs facilitates the release of several neurotransmitters, including glutamate, GABA and norepinephrine (Wonnacott, 1997; Wonnacott et al., 1990). All of them are known to contribute to the development of synaptic plasticity within the hippocampus (Placzek et al., 2009). The mechanism of facilitation involve nAChR mediated increases in presynaptic calcium concentration, which can be accomplished either directly via nAChRs high permeable to calcium (i.e. α7* or α5* nAChRs) or through depolarization and subsequent activation of VGCC. nAChRs located on soma and dendritic sites mediate nicotinic responses in the hippocampus, and β2* and α7* nAChRs subtypes have been demonstrated to be important mediators of these responses (Placzek et al., 2009).Within the hippocampus α3β4* nAChRs are located on glutamatergic terminals that synapse onto stratum radiatum interneurons and exert a powerful control on their resting excitability (Alkondon et al., 2011). Moreover, α3β4* nAChRs are localized on the soma of GABA interneurons and on axon terminals of parvalbumin-positive cells, where they mediate the release of GABA via low voltage-gated calcium channels (of T type) and calcium-induced calcium release (Tang et al., 2012). The α5 subunit expression predominates on stratum pyramidale of CA1 (Winzer-Serhan and Leslie, 1997) and may be an important candidate in regulating processes related to synaptic plasticity through increases in calcium permeability. Activation of nAChRs located on GABAergic interneurons can have important circuit level effects by either inhibiting or disinhibiting pyramidal neurons (Alkondon and Albuquerque, 2001; Alkondon et al., 1996; Ji and Dani, 2000). The activation of nAChRs located on interneurons contributes to the setting of the cooperative temporal framework that provides the basis for high cognitive functions (Rezvani and Levin, 2001)(Fig. 8).

Interestingly, nAChRs subunits are transiently expressed in the hippocampus during critical periods of development (Winzer-Serhan and Leslie, 1997) suggesting that cholinergic signalling participates in establishment of proper hippocampal morphological features and synaptic connectivity. In fact, abnormal activation of the cholinergic system during development (i.e. if smoking occurs during pregnancy) results in long-lasting consequences in the hippocampal morphology and synaptic

function (Roy and Sabherwal, 1994; Roy and Sabherwal, 1998; Roy et al., 2002; Wang et al., 2011). Importantly, these alterations correlate with cognitive impairment in later live (Weissman et al., 1999).

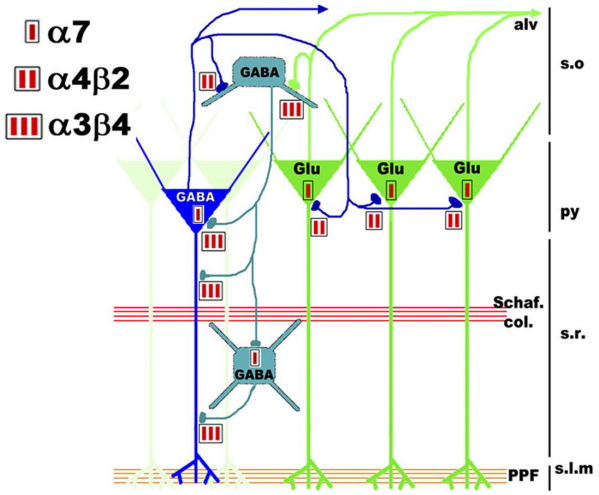


Figure 8. Nicotinic receptor modulation of hippocampal inhibitory circuitry. In the pyramidal layer (py) there are the excitatory pyramidal neurons that are glutamatergic (GLU; green) and pyramidal associated interneurons that are GABAergic (GABA; dark blue). These interneurons extend dendrites both in the direction of the stratum radiatum (SR) where they interact with Schaffer collaterals (Schaf.Col.) and terminate in the stratum lacunosum moleculare (SLM) to interact with perforant path fibers. The majority of nAChRs on these neurons are of the type I (α7) subtype, which can also be located on some principal excitatory neurons. Axons, which also express type II (α4β2) nAChRs, extend from interneurons to interact with many excitatory neurons and other interneurons. In some cases, they can extend to other hippocampal fields via the alveus (alv). Other inhibitory interneurons expressing nAChRs (light blue) are located in the SR and stratum oriens (SO). The SR interneurons often express nAChRs of the types I and II. Type III (α3β4β2) nAChRs are present on glutamate axons innervating SR interneurons and possibly other interneurons. Adapted from *Physiol. Rev 89. 73-120*. (Albuquerque et al., 2009).

Currently it is unclear how hippocampal synaptic and structural plasticity is affected by nicotine addiction or to what extent alterations in hippocampal function are factors of vulnerability to nicotine addiction. Even less is know about the consequences of a deregulation in α5, α3 and β4 nAChRs expression in hippocampal structure and functionality.

HYPOTHESIS AND OBJECTIVES

2. HYPOTHESIS AND OBJECTIVES

2.1.HYPOTHESIS

The magnitude of an individual's vulnerability to develop addiction on nicotine has a strong genetic influence. The *CHRNA5/CHRNA3/CHRNB4 (CHRNA5/A3/B4)* genomic cluster has been associated to nicotine dependence in human genetic studies but the specific mechanisms underlying this genetic vulnerability are not established. The effects of initial exposure to nicotine are a good predictor of later tobacco addiction in life supporting that sensitivity to nicotine depends on neuroplasticity in brain circuits that are involved both in reward and learning processes. Thus, our working hypothesis proposes that a deregulated expression of the α5, α3 and β4 nAChRs subunits increases sensitivity to nicotine, and nicotine-enhancement of learning process, driven by neuroplastic changes in specific brain regions.

2.2.OBJECTIVES

To prove our hypothesis we had to determine the role of the α5, α3 and β4 subunits of the nAChRs in nicotine addiction. To this end, and since human studies suggest that overexpression of the cluster subunits is possibly underlying increased nicotine sensitivity, we used a genetically modified mouse model overexpressing the human cluster *CHRNA5/A3/B4* (Tg*CHRNA5/A3/B4*). We studied the consequences of overexpression of the cluster on the sensitivity to nicotine and on reward and learning processes. To complete the picture, the work addressed cellular and molecular responses to nicotine including neuroplasticity in specific brain regions.

The concrete objectives have been to characterize how overexpression of the cluster *CHRNA5/A3/B4* modifies:

1. Sensitivity to some pharmacological effects of nicotine such as nicotine-induced hypolocomotion and seizures.

2. Cognitive domains associated to drug addiction, including working memory/attention, behavioural inhibition and contextual associations, in basal situation and upon acute and chronic nicotine administration.

3. The structure and function of the hippocampus, as an important substrate of addiction, in basal situation and after nicotine administration.

4. The nicotine addictive profile, focusing on nicotine self-administration, tolerance and withdrawal.

MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1. ANIMALS

We have used transgenic mice overexpressing the human genomic cluster *CHRNA5/A3/B4* (Tg*CHRNA5/A3/B4*) (Gallego et al., 2011)(generated as described in detail in supplementary methods). Transgenic line was maintained in hemizygosity by crossing with B6/SJL females to obtain transgenic mice. All experiments were performed using mice from the F21–F25 generation to attenuate littermate's genetic differences. We have used male mice in order to avoid female estrous cycle variations. Non-transgenic (wild type, WT) littermates served as controls. Male adult WT and Tg*CHRNA5/A3/B4* mice (2-4 months) were group-housed (2-5 animals per cage) in standard macrolon cages (40 cm long 625 cm wide 620 cm high) under a 12-h light/dark schedule (lights on 08:00 to 20:00) and controlled environmental conditions of humidity (50-70%) and temperature (22 ± 2ºC). Food and water were available *ad libitum.* All experimental procedures were approved by the local ethical committee (CEEA-PRBB), and met the guidelines of the local (Catalan law 5/1995 and Decrees 214/97, 32/2007) and European regulations (EU directives nº 86/609 and 2001-486) and the Standards for Use of Laboratory Animals nº A5388-01 (NIH). The CRG is authorized to work with genetically modified organisms (A/ES/05/I-13 and A/ES/05/14).

3.2. CHARACTERIZATION OF THE PHARMACOLOGICAL EFFECTS OF NICOTINE IN Tg*CHRNA5/A3/B4* MICE

The first part of the Thesis addressed how overexpression of the cluster *CHRNA5/A3/B4* altered sensitivity to nicotine. To this aim, the pharmacological effects of nicotine on locomotor activity were investigated. Additionally, diverse pharmacological tools were used to elucidating the cholinergic vs. non-cholinergic mechanisms underlying the hypersensitivity to nicotine-induced seizures previously detected in Tg*CHRNA5/A3/B4* animals (Gallego et al., 2011). Finally, the brain regions activated when nicotine-induced seizures were investigated using c-Fos expression analysis.

3.2.1. Nicotine-induced hypolocomotion

The effects of nicotine in locomotion were measured in activity cages (LE 881 Panlab SL., Spain) that consisted of an arena (45 cm long x 45 cm wide x 30 cm high) connected to infrared detection photobeams for analysis of horizontal activity. The test was performed under low non-aversive lighting conditions (50 lux.) to avoid stressful stimuli. Total distance travelled during 10 minutes was recorded using the Actitrack software (Panlab SL., Spain).

Experiment 1

The acute effects of nicotine were examined in randomly assigned groups of WT and Tg*CHRNA5/A3/B4* mice that received an acute subcutaneous (s.c.) injection (0.1 ml/10g body weight) of either saline (NaCl 0.9%) or nicotine hydrogen tartrate (0.5 and 1 mg/kg; Sigma, St.Louis, MO) dissolved in saline, 5 minutes prior the test (n = 9-11 mice per group).

Experiment 2

The effects of chronic nicotine on locomotor activity were analyzed in WT and Tg*CHRNA5/A3/B4* mice implanted with osmotic minipumps filled with either saline or nicotine hydrogen tartrate (10 mg/kg/day) dissolved in saline for 7 days (see "minipump implantation" section in supplementary methods) (n = 12-14 mice per group).

3.2.2. Nicotine-induced seizures

a. Cholinergic and non-cholinergic mechanisms

To determine whether nicotine-induced seizures were mainly induced by cholinergic activation, we administered mecamylamine, a non-competitive nAChRs antagonist, prior to nicotine, to study if we could prevent seizures. Besides, the possible involvement of the glutamatergic system was determined by injecting either MK-801, a non-competitive antagonist of the NMDA receptors or CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), a competitive antagonist of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors prior to nicotine

administration. Finally, the participation of the DAergic system was examined by giving prior to nicotine administration an antagonist for the D1 receptors, SCH-23390.

Mice received an acute intraperitoneal (i.p.) injection (0.1ml/10g body weight) (pre-treatment) of either saline, mecamylamine (0.5 mg/kg, Sigma, St.Louis, MO), MK-801 (0.5 mg/kg, Sigma, St.Louis, MO), CNQX (0.1 mg/kg, Tocris, Park Ellisville, MO) or SCH-23390 (0.1 mg/kg, Sigma, St.Louis, MO) dissolved in saline. The doses of all antagonists and time between drugs were selected according to (Damaj et al., 1999). After 10 minutes, mice received one s.c. injection (0.1 ml/10g body weight) of nicotine hydrogen tartrate (6, 12 or 24 mg/kg) dissolved in saline and were immediately placed in a transparent plastic cylinder (30 cm diameter, 50 cm high) for observation for 5 min. Nicotine induced seizures were scored according to (Franceschini et al., 2002) with slight modifications: 0 = no obvious effects; 1 = locomotor alterations; 2 = tachypnea, tremors and back arching; 3 = rapid movements of the legs (myoclonic seizures); 4 = complete loss of righting reflex and clonic seizures; 5 = abrupt extension of the limbs (tonic seizures); 6 = death. Each mouse received only one pharmacological combination (n = 8-14 mice per group).

Brain activation pattern after nicotine-induced seizures: c-Fos expression analysis

c-Fos protein expression was used to compare the brain activation pattern after an acute high dose of nicotine in WT and Tg*CHRNA5/A3/B4* mice. Mice received an acute s.c. injection (0.1 ml/10g body weight) of either saline or nicotine hydrogen tartrate (6 mg/kg) dissolved in saline and 120 minutes after injection they were sacrificed for c-Fos expression analysis. Due to the increased sensitivity to nicotine in Tg*CHRNA5/A3/B4* mice, 6 mg/kg nicotine elicited clonic/tonic seizures in transgenic animals, but not in their WT littermates. For this reason, a second group of WT mice was added that received a dose of nicotine (24 mg/kg) able to induce seizures (n = 3-4 mice per group). c-Fos expression was determined by immunofluorescence on tissue preparations (see protocol in section 3.5.4.) in brain regions involved in limbic seizures [medial habenula (MHb, Bregma -0.82 mm to -2.30 mm); lateral habenula (LHb, Bregma –1.22 mm to -1.70 mm); piriform cortex (Bregma -1.06 mm to – 2.18mm); lateral entorhinal cortex

(Lent, Bregma -2.75 mm to -3.1 mm); ventral tegemental area (VTA, Bregma - 2.92 mm to -4.04 mm); hippocampus (dorsal CA1, CA3 and DG subregions, Bregma -1.06 mm to – 2.18 mm); locus coeruleus (LC, Bregma -5.27 mm to -5.58 mm)] (Franklin and Paxinos, 1996). In the VTA and LC, double immunofluorescence against c-Fos and Tyrosine Hydroxylase (TH) (limiting enzyme for the synthesis of catecholamines, including dopamine and norepinephrine for the VTA and LC, respectively) was performed to delimitate the anatomical boundary of these regions.

b. Sensitivity to other pro-convulsive agents

The sensitivity to NMDA, a NMDARs agonist or to pentylenetetrazole (PTZ), a $GABA_A$ receptor antagonist was investigated in WT and Tg*CHRNA5/A3/B4* mice. Seizures induced by these pro-convulsive agents were scored according to two different seizure-rating scales due to differences in molecular mechanism and seizure manifestation. Seizures induced by NMDA, which caused directly stimulation of the glutamatergic system, were scored in accordance to the Racine scale (Racine et al., 2003) with slight modifications: 0 = no behavioural alteration; 1 = immobility, mouth and facial movements, facial clonus; 2 = head nodding, forelimb and/or tail extension, rigid posture; 3 = limb clonus, repetitive movements (myoclonic); 4 = rearing, falling and clonic seizures; 5 = tonic seizures, jumping; 6 = death. On the other hand, seizures induced by PTZ, which caused an inhibition of GABA receptors, were scored in accordance to the Gilbert scale (Gilbert, 1988) with slight modifications: 0 = no behavioural alteration; 1 = immobility, twitching of eyes and ears; 2 = head or neck clonus; 3 = myoclonic jerks (MCJ); 4 = prolonged MCJ, forelimb clonus; 5 = uni- or bilateral forelimb clonus enduring for more than 10 seconds; 6 = death. For each drug, the sum of all seizure episodes manifested by the animal along the observation period resulted in a seizure score. The percentage of animals that presented generalized clonic-tonic seizures was also registered.

Experiment 1

Mice received an acute i.p. injection (0.1 ml/10g body weight) of NMDA (Sigma, St. Louis, MO; 110, 170, 190 or 210 mg/kg) dissolved in saline and were immediately

placed in a transparent plastic cylinder (30 cm diameter, 50 cm high) to be observed for 30 min (n = 7-10 mice per group).

Experiment 2

Mice received an acute i.p. injection (0.1 ml/10g body weight) of PTZ (Sigma, St.Louis, MO, 50 mg/kg) dissolved in saline and were immediately placed in the mentioned plastic cylinder for 30 minutes observation (n = 7-8 mice per group).

3.3. CHARACTERIZATION OF THE COGNITIVE PROFILE IN Tg*CHRNA5/A3/B4* MICE

One of the main objectives of this Thesis project is to determine how the overexpression of the human gene cluster *CHRNA5/A3/B4* affected different aspects of cognitive performance related to addiction. The cognitive tests performed investigated various neurobehavioral traits associated to vulnerability to nicotine addiction and included the novel object discrimination and recurrent behaviour tasks (working memory/attention), the passive avoidance paradigm (behavioural inhibition), the Morris Water Maze test (cognitive flexibility), and the pure contextual fear-conditioning paradigm (contextual associations). Additionally, how the administration of nicotine either acutely or chronically modulated these cognitive capabilities was also evaluated.

3.3.1. The novel object discrimination task

Apparatus

Novel object discrimination was examined on an open-field that consisted of a rectangular arena (70 cm long x 70 cm wide x 30 cm high) made of Plexiglas. The walls and floor of the arena were covered with red plastic and surrounded by curtains to avoid the influence of external stimuli during the experiment. The task was performed under low non-aversive lighting conditions (50 lux). An overhead camera connected to the video-tracking software System Motor Activity Record and Tracking (SMART, Panlab

SL., Spain) was used to monitor the animal's behaviour and allowed to register time spent, travelled distance and speed in each zone of the maze.

The experimental design included three sessions:

Habituation session Animals were habituated for 10 minutes to an arena containing two objects located on the centre of the open field. The objects used in the habituation session were different between them and from the ones used during the familiarization and test sessions.

Familiarization and test sessions After 24 hours, in the familiarization session, two identical objects were located on the centre of the arena. The animal was placed into the centre of the arena and allowed to explore the objects for 10 minutes. After a delay of 1 hour (test session), the animal was placed again in the arena for 5 minutes and was presented with two objects, one of which was the same used in the familiarization session and the other a novel one. Discrimination index was calculated as time exploring the novel object – time exploring the familiar object / total time of exploration *100.

The position of the objects in the familiarization and test sessions was counterbalanced between animals. All the objects used were plastic made and induced similar exploration levels. The arena and objects were deeply cleaned between the animals to avoid olfactory cues. All measures of exploration were registered manually by an experimenter blind to genotype and treatment. Exploratory behaviour was defined as the animal directing its nose towards the object at a distance of < 2 cm. Sitting on or resting against the object was not considered as exploration. Subjects failing to complete a minimum of 20 seconds of exploration during the familiarization phase were excluded for posterior analysis.

Experiment 1

Novel object discrimination was examined in WT and Tg*CHRNA5/A3/B4* littermates in basal situation (n = 10-12 mice per group).

Experiment 2

The acute effects of nicotine on object discrimination were evaluated in mice that received a s.c. injection (0.1 ml/10g body weight) of either saline (NaCl 0.9%) or nicotine hydrogen tartrate (0.1 mg/kg, Sigma, St Louis, MO) dissolved in saline, 5 minutes prior the familiarization phase (n = 14 mice per group).

Experiment 3

The chronic effects of nicotine on object discrimination were analyzed in mice implanted with Alzet osmotic minipumps filled with either saline (NaCl 0.9%) or nicotine hydrogen tartrate (10 mg/kg/d, Sigma, St Louis, MO) dissolved in saline (see supplementary methods). The habituation session was carried out 4 days after minipump implantation and the familiarization and test sessions 24 hour later, when mice had received the chronic treatment for 5 days (n = 10-12 mice per group).

3.3.2. The recurrent behaviour task

Apparatus

The apparatus and testing conditions were the same as those of the novel object discrimination task. To assess recurrent exploration, mice were presented with two similar objects located on the centre of an open field and allowed to freely explore them for 10 min. The frequency each animal explored the same object one, two, three or more consecutive times (repeated explorations) was registered in WT and Tg*CHRNA5/A3/B4* mice (n = 12-13 mice per group).

3.3.3. The passive avoidance paradigm

Apparatus

The passive avoidance paradigm consisted of a circular platform (3 cm diameter) located on the centre of an electrified grid (Panlab SL., Spain). Mice were placed on the small platform and when they stepped down they received a foot shock (0.6 mA). The latency to step down from the platform was automatically registered. The apparatus was deeply cleaned between animals.

The paradigm comprised three sessions:

Acquisition session Mice were placed on the small platform and when they stepped down they received a 2 seconds 0.6 mA foot shock.

Test and retest sessions 24 hours and 7 days after the training session mice were placed again on the platform and the latency to step down was automatically measured. In the test and retest sessions the cut-off time (maximum step down latency) was set at 300 seconds. In the test session, mice received again a foot shock (0.6 mA) to avoid behavioural extinction for the retest session.

Experiment 1

The paradigm was conducted in WT and Tg*CHRNA5/A3/B4* littermates in basal situation (n = 22-25 mice per group).

Experiment 2

The acute effects of nicotine on behavioural inhibition were examined in mice that received a s.c. injection (0.1 ml/10 g body weight) of either saline (NaCl 0.9%) or nicotine hydrogen tartrate (0.1 mg/kg, Sigma, St Louis, MO) dissolved in saline, 5 minutes prior to the training session (n = 17 - 21 mice per group).

3.3.4. The Morris Water Maze test

Apparatus

The Morris Water Maze (MWM) test consisted of a circular tank (120 cm diameter, 100 cm high) filled with opaque water (with non-toxic white paint) and maintained at 21 ± 2ºC. A removable circular platform (13 cm diameter) made of Plexiglas was located inside the pool. The apparatus was surrounded by curtains so no external stimuli could be seen during the experiment and various distal cues, some of them three-dimensional, were located along the inner part of the curtains. The test was performed under low non-aversive lighting conditions (50 lux). An overhead camera connected to video-tracking software (SMART, Panlab SL., Spain) was used to monitor the animal's behaviour. Latency to reach the platform, total distance travelled, speed and time in zones were recorded for posterior data analysis.

The test comprised 10 sessions:

Pre-training session (P) On the first day, mice performed a familiarization session in order to acquire the procedural aspects of the task. The platform was visible and located on the centre of the pool.

Acquisition sessions (A1-A5) The visuospatial learning, mice were trained to locate the hidden platform situated on the north-east (NE) quadrant of the pool, for 5 consecutive days. The first acquisition session started 3 hours after the last trial of the pre-training session.

Removal session (RM) On day 5, and 3 hours after completion of the last trial of the acquisition session 5, mice were subjected to a probe trial, in which the platform was removed and each mouse was allowed to freely swim in the absence of the platform for 60 seconds. This task evaluated visuospatial reference memory.

Cued session (C) On day 6, all trained mice received a cued session, spatial cues were removed and the platform was flagged and located on the NE quadrant. This session was fundamental to detect differences in swimming or visual abilities, or emotional alterations.

Reversal session (RV1-RV2) On Day 6, and 3 hours after completion of the last cued trial, mice received two reversal sessions for 2 consecutive days, were the platform was hidden and moved to the SW quadrant of the pool. These two sessions addressed cognitive flexibility.

In every session each mouse was placed facing the outer edge of the pool and was given four daily trials (30 minutes inter-trial interval) from four peripheral releasing points corresponding to the North, East, South and West, randomly assigned along the different sessions. Mice that did not find the platform in a 60 seconds trial were guided to the platform by the experimenter. After each trial, all mice were allowed to remain on the platform for 15 seconds and placed back to their home-cages. In the removal session only one trial session was given, mice were situated on the centre of the pool and allowed to freely swim for 60 seconds. The water was vigorously stirred between animals to remove olfactory cues.

Visuospatial learning and cognitive flexibility were assessed in WT and Tg*CHRNA5/A3/B4* mice surgically implanted with Alzet osmotic minipumps filled with either saline (NaCl 0.9%) or nicotine hydrogen tartrate (10 mg/kg/d, Sigma, St Louis,

MO) dissolved in saline. The test started 24 hours after minipump implantation (see Supplementary Methods) (n = 13-15 mice per group).

3.3.5. The pure contextual fear conditioning paradigm

Apparatus

The fear conditioning apparatus consisted of a transparent Plexiglas operant chamber (15 cm long x 5 cm wide x 30 cm high) situated inside a sound-attenuating box that produced a white noise level of 60 dB and was slight illuminated. The grid floor was made of stainless steel rods (4 mm diameter) spaced 10 mm and through which scrambled electric shock could be delivered. All sub-tests were analyzed by the aid of the Startle-freezing program (Panlab SL., Spain).

The paradigm comprised two sessions:

Acquisition session Mice were placed inside the conditioning chamber and allowed to habituate for 120 seconds. Then, five electric shocks (0.2 mA, 2 second duration) were presented at 120, 150, 210, 240 and 255 seconds after the session started.

Test session 24 hours after the acquisition session, mice were placed again in the conditioning chamber for 120 seconds.

Freezing was defined as the lack of movement except of respiration and was accounted by the software when the animals' movement was reduced below a specific threshold. The floor trays and shock bars were deeply cleaned between sessions. In the acquisition session, percentage of freezing was calculated during the 2 minutes baseline period before exposure to the shock (basal) and also 15 seconds after receiving the foot shock for each trial presented. In the test session, freezing time was calculated during the 120 seconds.

Contextual memory was assessed in WT and Tg*CHRNA5/A3/B4* littermates implanted with Alzet osmotic minipumps filled with either saline (NaCl 0.9%) or nicotine hydrogen tartrate (10 mg/kg/d, Sigma, St Louis, MO) dissolved in saline. The acquisition session was performed when mice had received 5 days of treatment and the test session 24 hours later, when they were chronically treated for 6 days (see supplementary methods) (n = 13-16 mice per group).

3.4. CHARACTERIZATION OF THE HIPPOCAMPUS IN Tg*CHRNA5/A3/B4* MICE

We explored if overexpression of the cluster *CHRNA5/A3/B4* could alter the structure and functional connectivity of the hippocampus. To dig into this hypothesis, morphological parameters such as the hippocampal layering, and the structural complexity and dendritic spine density in hippocampal pyramidal neurons were evaluated. Moreover, electrophysiological properties were also investigated in hippocampal slices. Additionally, we analyzed the effects of nicotine administration on these parameters in WT and Tg*CHRNA5/A3/B4* mice. To better understand the possible changes of hippocampal structural plasticity when the cluster *CHRNA5/A3/B4* is overexpressed hippocampal primary cultures were used.

3.4.1. Morphological study of the hippocampus

To study general morphological features of the hippocampus, Tg*CHRNA5/A3/B4* male mice were crossed with *Thy1-Yellow Fluorescent Protein (YFP)* heterozygous female mice (Jackson Laboratories) to generate WT and Tg*CHRNA5/A3/B4* mice that expressed *YFP* sparsely in subsets of pyramidal neurons. This genetic tool allowed measuring the different layers of the hippocampus and quantifying the number of neurons in pyramidal layers (dorsal CA1, CA3 and DG subregions, Bregma -1.06 mm to – 2.18 mm). The morphometrical parameters of hippocampal strata were analyzed in *Thy1-YFP* WT and *Thy1-YFP* Tg*CHRNA5/A3/B4* mice implanted with osmotic minipumps delivering either saline (NaCl 0.9%) or nicotine hydrogen tartrate (10 mg/kg/d, Sigma, St Louis, MO) dissolved in saline, for 7 days (see supplementary methods)(n = 4-6 mice per group). Briefly, Mice were deeply anesthetized with isofluorane, and perfused with 0.1M phosphate buffer saline (PBS) followed by chilled 4% paraformaldehyde (PFA, Sigma, St Louis, MO), the brain was postfixed in 4% PFA and cryoprotected in 30% sucrose. A vibratome (Leica, Germany) was used to cut coronal sections (150 μm thick) and slices were washed and mounted on gelatine-coated slides with aqueous solution (Mowiol).

Image acquisition and analysis

Images at 20x were taken at different *x*, *y* and *z* coordinates to visualize the entire hippocampus, using the SP5 Confocal Microscope (Leica, Germany) connected to the LAS AF software (Leica, Germany). Each image was a *z* series projection of approximately 10-12 stacks, and taken at 5 μm depth intervals. A composition of the different images was automatically done by the software. The length of each hippocampal strata and the number of somas in the pyramidal layers were manually calculated with Image J software.

3.4.2. Structural complexity of pyramidal neurons

Primary cell cultures

Primary cultures of hippocampal neurons were obtained from WT and mice and Tg*CHRNA3/A5/B4* mice at embryonic day (E) 17.5-18.5. Pregnant females were sacrificed by cervical dislocation; the embryos were extracted and placed inside a sterile Petri dish maintained on ice. The brain was removed from the skull and a tail biopsy was kept in ice for subsequent genotyping. Briefly, meninges were removed and the hippocampi dissected under magnifying glass; the hippocampi were kept on a drop of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, US) placed in a sterile Petri dish on ice. Neurons were mechanically dissociated using a sterile scalpel and they were collected in 2 ml of DMEM in a new sterile 15 ml tube. Neurons were mechanically separated by softly pippeting and centrifuged at 1030 rpm for 5 min. The supernatant was removed and the neurons were resuspended in 0.5 ml of culture medium Neurobasal (Invitrogen, US) supplemented with 2% B27 factor (Invitrogen, US), 1% Glutamax (Invitrogen, US), 0.5% penicillin/streptomycin (Invitrogen, US) (NB+++) and also 10 % Horse Serum Heat Inactivated (Invitrogen, US). Neuronal density was determined using a Neubauer chamber and neurons were plated at the appropriate dilution in NB+++ medium with serum. Cells were then incubated inside the CO_2 incubator under culture conditions (37ºC and 5% CO_2). 24 h after plating the serum was removed and substituted by NB+++ to avoid massive glial proliferation. Before plating, all the plates were previously treated with 4μg/ml Ethylene Imine Polymer solution PEI (Fluka) in sterile water for 12h at 37ºC to facilitate cell adhesion. The medium was changed every 3 days. At day-in-vitro (DIV) 7, when neurons had become polarized and

start to mature, on a half of the plate the medium was replaced by fresh medium and on the other half it was replaced by fresh medium with nicotine (10μM, Sigma, St.Louis, MO). Neurons were exposed to nicotine treatment for 2 days, until DIV9, when all the analyses were acquired.

Cell culture viability: MTT assay

Neurons were seed on a 96-well plate at a density of $1x10^4$ cells per well, nicotine (10μM, Sigma, St Louis, MO) was added to the culture medium at DIV7, as described above. At DIV9, neurons were incubated with MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazlium bromide) (500 μg/ml, Sigma, St Louis, MO) in 0.1M PBS for 30 min at 37 ºC and under dark conditions. After incubation the medium was removed and the formazan dye was extracted using 100% Detergent Sodium Dodecyl Sulfate (DMSO, Sigma, St Louis, MO). The absorbance was determined using a microplate reader at 550 nm (n = 6 experiments per group).

Image acquisition and analysis

Neurons were seed at a density of $5x10^4$ cells per well in round glass coverslips placed on a 24-well plate. At DIV5, cultures were transfected with a plasmid containing *enhanced green fluorescent protein* (*EGFP)* driven by the *Thy*1 promoter (designed and kindly provided by Dr. G. Ramakers), by means of Lipofectamine 2000 (Invitrogen, US) and following manufacturer instructions. Arborisation of pyramidal neurons derived from WT and Tg*CHRNA5/A3/B4* cultures was examined in basal situation but also after 48 hours of nicotine treatment. At DIV7, half of the plates were incubated with fresh medium and the other half with nicotine (10 μM, Sigma, St.Louis, MO), for 2 days. At DIV9, cultures were fixed in PFA 4% for 20 minutes at RT, washed and mounted on slides with aqueous solution (Mowiol). Positive cells expressing *EGFP* were recorded in 3D image stacks with SP5 Confocal Microscope (Leica, Germany) connected to the LAS AF software (Leica, Germany). Only non-overlapping positive neurons expressing *EGFP* were used for morphological analysis. All images were acquired using a 63x objective generating a z-stack from 7-9 images taken at a 0.5 μm depth interval. Since the whole neuron was not observed in a single field, a composition of different fields was automatically done by the software, to visualize the entire neuron.

The structural complexity of pyramidal neurons was determined by means of the Sholl analysis, which quantifies the number of intersections arising from concentric circles with increasing radii extending from the soma (Sholl, 1953). Sholl analysis plugging of Image J software was used to determine the number of intersections in concentric circles of radii that incremented by 10 μm from the soma. (n = 28 – 30 neurons per group; note that neurons derived from at least 3 independent experiments).

3.4.3. Dendritic spine density in pyramidal neurons

Dendritic protrusions based on their morphology were classified into three major types: stubby (type-I), mushroom (type-II), and thin (type-III) spines (Bourne and Harris, 2008). Each spine subtype was classified based on the L/N and H/N ratio where L was spine length, H was the maximum head width, and N was the maximum neck width. Following these criteria, stubby spines had a length that was similar to the diameter of the neck and similar to the diameter of the head (L≈N≈H); mushroom spines had a greater H/N ratio (H>N); and the length of thin spines was much grater than their neck diameter (L>>N). Also filopodia density was evaluated. Filopodia represented immature spines and use not to be present in the adult mouse brain, they were morphologically distinct from the other spines subtypes in that the length was greater than 2 μm and much grater than the diameter.

Image acquisition and analysis

Spine density analysis was conducted in neurons obtained from WT and Tg*CHRNA5/A3/B4* hippocampal primary cultures transfected with a plasmid containing *EGFP* driven by the *Thy*1 promoter. At DIV7, cultures were incubated with fresh medium or medium with nicotine (10 μM) for 2 days, from DIV7 to DIV9, as described above. Confocal images were obtained using the SP5 Confocal Microscope (Leica, Germany) connected to the LAS AF software (Leica, Germany). z-series stacks (7-9) taken at a 0.36 μm depth interval from images acquired at 63x with an optical zoom of 2.0 were subjected to three-dimensional reconstruction for posterior analysis. Spines were morphologically categorized as described (stubby, mushroom, thin or filopodia), and expressed as number of spines per 10 μm of dendritic length. Spine analysis was performed by the experimenter using Image J software in the 30 μm length region

proximal to the first branchpoint of pyramidal neurons (n = 25 dendrites per group; dendrites derived from at least 12 neurons from 3 independent experiments).

3.4.4. Electrophysiological properties of the hippocampus: Paired-pulse facilitation and long-term potentiation (LTP)

Hippocampal slices from adult WT and Tg*CHRNA5/A3/B4* mice were maintained in artificial cerebrospinal fluid (ACSF) (in mM): NaCl, 124; KCl, 2.5; $MgSO_4$, 1; $NaHPO_4$, 1.25; $CaCl_2$, 2.5; $NaHCO_3$, 26; and dextrose, 10 and was aerated with 95% O_2-5% CO_2 to a final pH of 7.4.). Evoked Field excitatory postsynaptic potentials (fEPSPs) were recorded in the stratum radiatum of dorsal hippocampus CA1 subregion (Bregma -1.06 mm to – 2.18 mm) in response to stimulation of the Schaffer collateral pathway. For each slice, after establishing a stable baseline, the paired-pulse facilitation was induced by a double-pulse (30 ms apart) stimulation protocol and it was evaluated by calculating the paired-pulse facilitation ratio (PPR) between the second ($fEPSP_2$) and the first ($fEPSP_1$) responses. After 20 minutes baseline registering (pulse at 0.03 Hz), LTP was induced by high-frequency stimulation (HFS) that consisted of 5 trains of 100 Hz (1 s) with 10 seconds intervals. LTP was registered for 60 minutes (pulse at 0.03 Hz). Recordings were digitized, acquired, and analyzed using a data acquisition interface and software from Cambridge Electronic Design (Spike2, Cambridge, UK). The effects of acute nicotine exposure were also evaluated. Slices were incubated for 20 minutes with nicotine (100 μM, Sigma, St.Louis, MO) dissolved in ACSF solution, washed with ACSF and immediately after, PPR and LTP were registered as recently described (n = 8-11 slices per group; slices derived from at least 4 mice per genotype) (detailed protocol for electrophysiological recordings in supplementary methods).

3.5.CHARACTERIZATION OF THE NICOTINE ADDICTIVE PROFILE IN Tg*CHRNA5/A3/B4* MICE

We inspected how overexpression of the cluster *CHRNA5/A3/B4* modulated specific aspects of nicotine addiction, such as nicotine self-administration (the two bottle choice paradigm), and nicotine-induced withdrawal and tolerance. Moreover, the

nicotine-induced activation of the VTA, the habenula (MHb and LHb), brain regions involved in the rewarding and/or aversive properties of nicotine were studied.

3.5.1. The two-bottle choice paradigm

Voluntary nicotine consumption and preference were examined in mice that had 24 hours access to two inverted 25 mL tubes with metal sippers placed on stainless steel cage tops. One tube always contained tap water and the other increasing concentrations of nicotine (Sigma, St. Louis, MO) in tap water. Mice had access to each nicotine concentration for 4 days with bottle positions alternating every two days. Food was distributed near both bottles to avoid food-associated tube preferences. Each morning, daily fluid intake was recorded from both bottles by measuring the level of the meniscus on the graduated drinking tube. Nicotine and water tubes placed on two empty cages allowed for the measurement of leakage and evaporation from the tubes. The average volume depleted from these "control" tubes was subtracted from the individual drinking volumes each day before data analysis. To obtain a measure of nicotine consumption that corrected for individual differences in mouse size, mg of nicotine consumed per kg of body weight per day were calculated for each mouse. Average nicotine consumption per day was calculated for each nicotine concentration. The nicotine preference ratio was calculated daily for each nicotine concentration as volume of nicotine solution consumed per total volume of fluid (water plus nicotine solution) consumed. Average nicotine preference per day was calculated for each nicotine concentration. Body weight was recorded simultaneously with each solution change.

Experiment 1

Nicotine consumption (mg/kg/d) and preference were measured as described above in WT and Tg*CHRNA5/A3/B4* mice when very low doses of nicotine were available in drinking water. Mice were habituated for 4 days to be individually housed and to the free choice of two bottles that contained tap water. On day 4 a choice was offered between tap water and increasing concentrations of nicotine (1 and 5 µg/ml in tap water) (n = 8-11 animals per group).

Experiment 2

Nicotine consumption (mg/kg/d) and preference were measured as described above in WT and Tg*CHRNA5/A3/B4* mice when a wide range of nicotine doses were available in drinking water. Mice were habituated for 4 days to be individually housed and to the free choice of two bottles that contained tap water. On day 4 a choice was offered between tap water and increasing concentrations of nicotine (10, 20, 35, 50, 100 and 200 μg/ml in tap water). After finishing voluntary nicotine consumption mice were tested for preference to sweetened or bitter taste water, in order to exclude that the overexpression of the cluster affected taste properties. Mice were given two tubes containing water for 4 days and then were given plain water in one tube and saccharin (0.2 % solution in tap water) in the other pipette, using a protocol similar to that described for nicotine consumption. After 4 days exposure to the saccharin solution, it was replaced by a quinine solution (0.5 mM) and mice had 4 days of access to quinine vs. water. Consumption (mg/kg) and preference for each solution were calculated as described for nicotine (n = 13-16 animals per group).

Experiment 3

The third experiment explored whether an acute exposure to a very high dose of nicotine was able to modify the subsequent behaviour of oral nicotine self-administration. This experiment was addressed to investigate possible plastic changes derived from a previous exposure to the drug, and which may affect the pattern of nicotine intake. Mice were habituated to be individually housed and to free choice of two bottles filled with water. On day 4, mice were exposed to a high dose of nicotine (10^3 μg/ml) for a short-time period (12h), and returned to tap water for 3 days. On day 8 they started the choice of increasing nicotine concentrations (10, 20, 35, 50, 100 and 200 μg/ml in tap water). Nicotine consumption (mg/kg/d) and preference were measured as described above. After finishing voluntary nicotine consumption mice were tested for preference to sweet or bitter tasting water as described in experiment 2 (n = 14 mice per group).

3.5.2. Nicotine-induced withdrawal syndrome

The appearance of somatic and affective signs of nicotine-induced withdrawal and withdrawal-induced hyperalgesia were evaluated in mice implanted with osmotic minipumps that infused for 7 days either saline (NaCl 0.9%) or nicotine hydrogen tartrate (10 mg/kg/day, Sigma, St. Louis, MO) dissolved in saline (see minipump implantation in supplementary methods). Nicotine-induced withdrawal syndrome was precipitated with a challenge with the nAChRs antagonist mecamylamine or spontaneously induced by minipump removal. For precipitated withdrawal, on the morning of day 7, mice received an acute i.p. injection (0.1 ml/10 g body weight) of mecamylamine (1 mg/kg, Sigma, St. Louis, MO) dissolved in saline, and withdrawal signs were measured immediately after antagonist injection (n = 5-6 mice per group). For spontaneously induced withdrawal, on day 7 the minipump was explanted under an O_2-isofluorane anaesthetic mixture and withdrawal data were collected 24 hours after removal (n = 12-15 mice per group).

a. Somatic signs

Somatic components of nicotine withdrawal were evaluated as previously described (Castane et al., 2002). Mice were individually placed in a transparent plastic cylinder (30 cm diameter, 50 cm high) and observed for 20 min. The number of grooming episodes, wet dog shakes, paw tremors, writhes and scratches that appear along the 20 minutes period were counted and each episode was given a score of 0.5. The presence of body tremor, ptosis, teeth chewing and piloerection received a score of 1 during each 5 minutes observation period along 20 minutes. The relative locomotor activity for each 5 minutes period was scored as 0 normal activity, 0.5 low activity and 1 inactivity. The sum of all the above stated assigns in the 20 minutes observation period provided a general withdrawal score for each animal.

b. Affective signs

After scoring the somatic signs of withdrawal, affective signs of nicotine-induced withdrawal were measured using the light and dark test for mecamylamine-precipitated withdrawal, and the elevated plus maze test for spontaneous withdrawal.

The light and dark test

The light and dark (L&D) test consisted of a small (15 x 20 x 25 cm) PVC compartment with black walls and black floor dimly illuminated (50 lux), connected by a 5 cm long tunnel to a large PVC compartment (30 x 20 x 25 cm) with white walls and floor, and intensely lit (500 lux). At the beginning of the 5-minutes session each mouse was placed on the light compartment facing the wall opposite to the tunnel. A video-tracking camera, connected to the System Motor Activity Record and Tracking (SMART, Panlab SL., Spain) software allowed registering the latency to enter to the dark compartment, time spent, number of entries, speed and distance in the different compartments.

The elevated plus maze test

The elevated plus maze (EPM) test consisted of a black Plexiglas apparatus with four arms (29 cm long x 5 cm wide) set in cross from a neutral central square (5 cm x 5 cm). Two opposite arms were delimited by vertical walls (closed arms) and the other two arms had unprotected edges (open arms). The maze was elevated 40 cm above the floor and placer under indirect light (100 lx). At the beginning of the 5-minutes session each mouse was placed in the central zone, facing one of the open arms. A video-tracking camera connected to the SMART software (Panlab SL., Spain) allowed registering time spent, number of entries, speed and distance in the different zones.

c. Hyperalgesia

Mice were placed on a hot plate at 52 ± 0.1ºC surrounded by a plastic cylinder (19 cm diameter, 19 cm high) to evaluate withdrawal-induced hyperalgesia. Two nociceptive responses: the latency to paw licking and the latency to jump were manually

recorded by the experimenter. A maximum latency of 180 s was set up to avoid tissue damage.

3.5.3. Nicotine-induced tolerance

Nicotine-induced tolerance is defined as the loss of potency with repeated administrations and is reflected by a shift to the right of the nicotine dose-response curve. The expression of tolerance was examined in WT and Tg*CHRNA5/A3/B4* mice that received an acute high dose of nicotine after being chronically treated with nicotine. Mice were implanted with osmotic minipumps filled with either saline or nicotine hydrogen tartrate (10 mg/kg/d) dissolved in saline (see supplementary methods). After 7 days of chronic treatment mice received an acute s.c. injection (0.1 ml/10g body weight) of nicotine hydrogen tartrate (6 mg/kg) dissolved in saline and were immediately placed in a transparent cylinder (30 cm diameter, 50 cm high) to be observed for 5 min. The appearance of seizures, latency to seizure and percentage of animals that experimented clonic/tonic seizures were registered as described above (n = 4-5 mice per group).

3.5.4. Nicotine-induced activation of the VTA, MHb and LHb: c-Fos expression analysis

Mice received one s.c. injection (0.1 ml/10 g body weight) of either saline (NaCl 0.9%) or nicotine hydrogen tartrate (0.5 mg/kg, Sigma, St. Louis, MO) dissolved in saline. 120 minutes after injection mice were deeply anaesthetized with isofluorane and perfused pericardially with 0.1M PBS, followed by chilled 4% PFA (n = 4 mice per group). Brains were extracted, postfixed with 4% PFA for 1 day, cryoprotected for 2 days in 30% sucrose and stored at -80ºC. The number of c-Fos positive nuclei in the VTA (Bregma - 2.92 mm to -4.04 mm), MHb (Bregma -0.82 mm to -2.30 mm) and LHb (Bregma –1.22 mm to -1.70 mm) was determined.

Immunofluorescence assays

Coronal frozen sections (40 μm) were obtained with a cryostat (Leica, Germany), washed in 0.1M PBS, permeabilized with 0.1M PBS 0.2% Triton X-100 (PBST)

(Sigma, St. Louis, MO), and blocked in 5% Foetal Bovine Serum (FBS) in PBST for 1h at room temperature (RT). The sections were then incubated with the corresponding primary antibodies (anti-c-Fos, 1:500, Santa Cruz Biotechnology plus anti-tyrosine hydroxilase, 1:4000, Sigma or anti-choline acetyltransferase, 1:1000, Santa Cruz Biotechnology) overnight at 4ºC, washed in PBST and incubated with the secondary fluorescently labelled antibodies (Alexa ®Fluor 488 and Alexa ®Fluor 555, Molecular Probes) in 1 % FBS in PBST for 1h at RT. After several washes, nuclei were stained with Hoechst; slices were mounted on slides and fixed with aqueous solution (Mowiol). Images obtained at 20x were acquired from coronal sections (40μm) using the SP5 Confocal Microscope (Leica, Germany) connected to the LAS AF software (Leica, Germany). Each image was a z series projection of approximately 5-7 stacks, and taken at 5 μm depth intervals. The number of c-Fos positive nuclei and the area delimiting each anatomical region were manually calculated with Image J software.

Immunohistochemistry assays

For immunohistochemistry to analyze c-Fos expression on tissue preparations, coronal frozen sections were obtained as described in the preceding paragraph, but after washing in 0.1M PBS the endogenous peroxidase activity was deactivated in 3% hydrogen peroxidase (H_2O_2), 10% Methanol in PBS for 20 min at RT under non-lighting conditions. After additional washes of 0.1M PBST slices were blocked in 5% FBS in PBST for 1h at room temperature (RT) and incubated with the corresponding primary antibody (anti-c-Fos, 1:500, Santa Cruz Biotechnology) in 1% FBS in PBST overnight at 4ºC. Slices were incubated with the biotinylated secondary antibody anti-rabbit (Vector Laboratories) in 1% FBS PBST for 1h at RT, washed and incubated in avidin-biotin peroxidase complex (ABC Elite; Vector Laboratories) during 2 hours at RT, under dark conditions. The colour reaction was developed with 0.05% 3,3-diamio-benzide-tetra-hydrochloride (DAB) and 0,1% H_2O_2 in PBS. The reaction was terminated with washes in 0.1M PBS, the slices were mounted in gelatine coated slides, nuclei were counterstained with cresyl violet and slices were dehydrated using an alcohol battery ending with xylol. An Olympus BX51 microscope connected to a videocamera and the CAST-GRID software was used to count positive c-Fos nuclei and to delimitate the corresponding areas.

3.6. STATISTICAL ANALYSIS

The statistical analysis was performed with the aid of Statistical Package for the Social Sciences (SPSS) software. For those experiments with only one independent variable (genotype), an independent-sample Student's T test was applied. For those experiments with two or more independent variables (genotype and treatment) the multivariate analysis of variance (MANOVA) was used. Only in cases in which a significant ($p < 0.05$) genotype x treatment interaction was found, then a Bonferroni *post hoc* comparison was performed between individual groups. Whenever the interaction between genotype and treatment was not significant, the Genotype effect or the Treatment effect was considered statistically significant when $p < 0.05$. For those experiments with repeated events giving consecutive data (curves), repeated measures ANOVA was applied, and considering significance in within factor effects and between-subject effect, this last being one independent variable (genotype) or more (genotype and treatment) if required. Again, if a significant genotype x treatment interaction was found, a Bonferroni *post hoc* comparison was applied between individual group curves, alternatively, the significant effect of genotype or treatment was considered (if $p < 0.05$).

Independent Sample T- Test (Genotype comparisons)

Significance	Symbol
$p < 0.05$	Φ
$p < 0.01$	ΦΦ
$p < 0.001$	ΦΦΦ

MANOVA / Repeated Measures ANOVA

	Symbol			
Significance	Comparison between genotype	Comparison between treatment	Genotype Effect	Treatment Effect
$p < 0.05$	Φ	*	&	Ω
$p < 0.01$	ΦΦ	**	&&	ΩΩ
$p < 0.001$	ΦΦΦ	***	&&&	ΩΩΩ

RESULTS

4. RESULTS

4.1.CHARACTERIZATION OF THE PHARMACOLOGICAL EFFECTS OF NICOTINE IN Tg*CHRNA5/A3/B4* MICE

Our previous results demonstrated increased nicotine binding sites in membrane preparations of Tg*CHRNA5/A3/B4* mice hippocampus and cerebral cortex (Gallego et al., 2012), with increased α3β4* nAChRs in brain regions where these subunits are endogenously expressed. The increased nAChRs binding sites lead to increased sensitivity to nicotine, as shown by the shift to the left of the dose-response for nicotine (Gallego et al., 2011). The first part of the Thesis addressed how the overexpression of the *CHRNA3/A5/B4* cluster altered the sensitivity to the pharmacological effects of nicotine by measuring locomotor activity. Additionally, several pharmacological tools were used to elucidate which mechanisms (cholinergic and non-cholinergic) underlie the hypersensitivity to nicotine-induced seizures observed in Tg*CHRNA5/A3/B4* animals. The brain regions activated upon nicotine-induced seizures were investigated using c-Fos protein expression analysis.

4.1.1. Nicotine-induced hypolocomotion

Experiment 1

The effects of acute nicotine administration on locomotor activity were analyzed in WT and Tg*CHRNA5/A3/B4* mice that received either saline or nicotine (0.5 or 1 mg/kg) 5 minutes before being placed inside the activity cages. Two-way ANOVA revealed that the effects of nicotine were different in Tg*CHRNA5/A3/B4* mice [genotype x treatment interaction, $F_{2,57} = 2.573$, $p < 0.05$]. At 1 mg/kg, locomotion was further decreased in Tg*CHRNA5/A3/B4* mice compared to their WT littermates, which significantly travelled less distance along the 10 minutes session ($p < 0.05$) (Fig. 1A).

Experiment 2

The effects of chronic nicotine on locomotor activity were also analyzed in mice implanted with osmotic minipumps delivering either saline or nicotine (10 mg/kg/d) for

7 days. Two-way ANOVA did not reveal genotype, or treatment effect, neither genotype x treatment interaction (Fig. 1B) indicating that the chronic nicotine treatment of 10 mg/kg/d for 7 days had no consequences on locomotor activity.

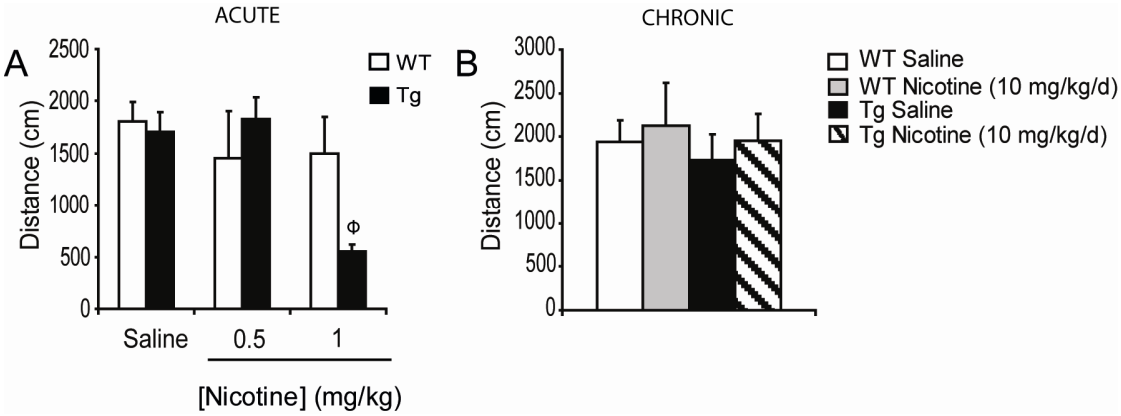


Figure 1. Tg*CHRNA5/A3/B4* mice show increased hypolocomotion at high acute doses of nicotine. **(A)** Distance travelled along 10 minutes session in WT (open) and Tg*CHRNA5/A3/B4* (filled) mice that received an acute injection of saline, 0.5 mg/kg or 1 mg/kg nicotine. N = 9-11 mice per group. **(B)** Distance travelled along 10 minutes session in WT mice chronically treated either saline (open) or 10 mg/kg/d nicotine (gray) and Tg*CHRNA5/A3/B4* mice chronically treated with saline (filled) or 10 mg/kg/d nicotine (striped), for 7 days. N = 12-14 mice per group. Data are expressed as mean ± S.E.M. Two-way ANOVA genotype x treatment interaction ($p < 0.05$). Genotype effect Φ $p < 0.05$.

4.1.2. Nicotine-induced seizures

a. Cholinergic and non-cholinergic mechanisms

Our previous work (Gallego et al., 2011) showed that overexpression of the cluster *CHRNA3/A5/B4* increases sensitivity to nicotine-induced seizures. In the first experiment, we injected saline or mecamylamine (0.5 mg/kg), a non-competitive antagonist of the nAChRs, 10 minutes before administration of nicotine (6, 12 and 24 mg/kg) to determine to what extent we could prevent seizures. Two-way ANOVA revealed significant genotype x pre-treatment interaction at the doses of 6 [$F_{1,51} = 5.711$, $p < 0.05$] and 12 mg/kg [$F_{1,32} = 63.250$, $p < 0.001$] (Fig. 2A). Bonferroni *post hoc* comparison between groups confirmed a significant increase in seizure score in Tg*CHRNA5/A3/B4* mice compared to their WT littermates, as previously observed (Gallego et al., 2011). Pre-treatment with mecamylamine significantly reduced seizure

score in Tg*CHRNA5/A3/B4* animals when nicotine was administered at 6 mg/kg ($p < 0.001$) and 12 mg/kg ($p < 0.001$). In WT animals pre-treatment with mecamylamine did not produce significant effects at the doses of 6 and 12 mg/kg since those only elicit seizures in Tg*CHRNA5/A3/B4* mice. When nicotine was administered at the dose of 24 mg/kg two-way ANOVA revealed a significant effect of pre-treatment [$F_{1,39} = 33.886$, $p < 0.001$] and genotype [$F_{1,39} = 11.081$, $p < 0.01$], but the interaction genotype x pre-treatment was not statistically significant. These results confirmed that overexpression of the cluster *CHRNA3/A5/B4* leads to hypersensitivity to nicotine-induced seizures and indicate that mecamylamine pre-treatment partially prevents seizure in both WT and Tg*CHRNA5/A3/B4* mice. Noticeably, in Tg*CHRNA5/A3/B4* mice, mecamylamine was only able to attenuate the clonic/tonic component of nicotine-induced seizures (see Table 1) but not other behavioural manifestations such as myoclonic seizures or back arching.

Damaj (Damaj et al., 1999) proposed that nicotine administration either directly or indirectly produces a rise in intracellular free calcium leading to the release of glutamate with consequent NMDARs activation and production of nitric oxide (NO), leading to seizures (Snyder and Bredt, 1991). The second experiment addressed whether hypersensitivity to nicotine-induced seizures observed in Tg*CHRNA5/A3/B4* mice could be blocked by pre-treatment with MK-801, a non-competitive antagonist of the NMDA receptors. Mice received an acute injection of saline or MK-801 (0.5 mg/kg) 10 minutes before administration of nicotine (6, 12 and 24 mg/kg). At the doses of 6 and 24 mg/kg two-way ANOVA demonstrated significant effect of genotype [$F_{1,47} = 96.195$, $p < 0.001$] and [$F_{1,39} = 57.015$, $p < 0.001$], respectively, but the genotype x pre-treatment interaction was not significant. However, pre-treatment with MK-801 completely attenuated seizures induced by 24 mg/kg nicotine in WT mice (Fig. 2B). At the dose of 12 mg/kg of nicotine two-way ANOVA yielded a significant genotype x pre-treatment interaction [$F_{1,31} = 4.947$, $p < 0.05$] (Fig. 2B). Bonferroni *post hoc* comparisons showed that pre-treatment with a NMDARs antagonist blocked seizures in WT mice as previously reported (Damaj et al., 1999), but it led to increased nicotine-induced seizures in Tg*CHRNA5/A3/B4* animals. MK-801 pre-treatment significantly increased seizure score in Tg*CHRNA5/A3/B4* mice ($p < 0.01$) and resulted in higher number of episodes of circling and jumping behaviour only in Tg*CHRNA5/A3/B4* mice (data not shown).

We thus asked whether nicotine hyperexcitability could occur through AMPA/kainate glutamatergic receptors. To explore this possibility, a separate group of mice were injected with either saline or CNQX (0.1 mg/kg), a competitive AMPA/kainate receptors antagonist, 10 minutes before nicotine administration at seizure producing doses (12 mg/kg in Tg*CHRNA5/A3/B4* and 24 mg/kg in WT mice). CNQX have been reported to be active against the tonic phase of generalized tonic-clonic seizures (Velisek et al., 1995). In our experiments, two-way ANOVA demonstrated that CNQX was not able to prevent nicotine-induced seizures neither in WT nor in Tg*CHRNA5/A3/B4* animals (Fig. 2D), thus excluding the participation of these receptors.

Finally, the possible involvement of the DAergic system was examined by using SCH-23390 (0.1 mg/kg), a D1 receptor antagonist. Previous in vivo pharmacological studies with SCH-23390, an antagonist of the D1 receptors, have shown that it is able to abolish generalized seizures evoked by the chemoconvulsant agent soman, an inhibitor of the cholinesterase enzyme (Bourne et al., 2001). Again, no significant effect of SCH-23390 in blocking seizures was detected in either genotype (Fig. 2C).

In summary, Tg*CHRNA5/A3/B4* mice presented seizures at all the doses of nicotine used, and the only pre-treatment that blocked seizures was mecamylamine. In WT mice, the only dose of nicotine that elicited seizures was 24 mg/kg, which could be blocked with mecamylamine and MK-801, but not with for D1 or AMPA/kainate receptors antagonists (Table 1).

		Pre-treatment				
Genotype	**Treatment**	**Saline**	**Mecamylamine (0.5 mg/kg)**	**MK-801 (0.5 mg/kg)**	**SCH-23390 (0.1 mg/kg)**	**CNQX (0.1 mg/kg)**
WT	**6 mg/kg**	0	0	0	nd	nd
	12 mg/kg	0	0	0	nd	nd
	24 mg/kg	43.75	0	11.11	28.57	50
Tg	**6 mg/kg**	70.58	14.28	83.33	nd	nd
	12 mg/kg	100	28.57	100	85.71	100
	24 mg/kg	100	62.5	100	nd	Nd

Table 1. Percentage of mice that displayed clonic/tonic seizures upon pre-treatment with cholinergic (mecamylamine), glutamatergic (MK-801 and CNQX) and DAergic (SCH-23390) antagonists. N = 8-16 mice per group. nd = not determined.

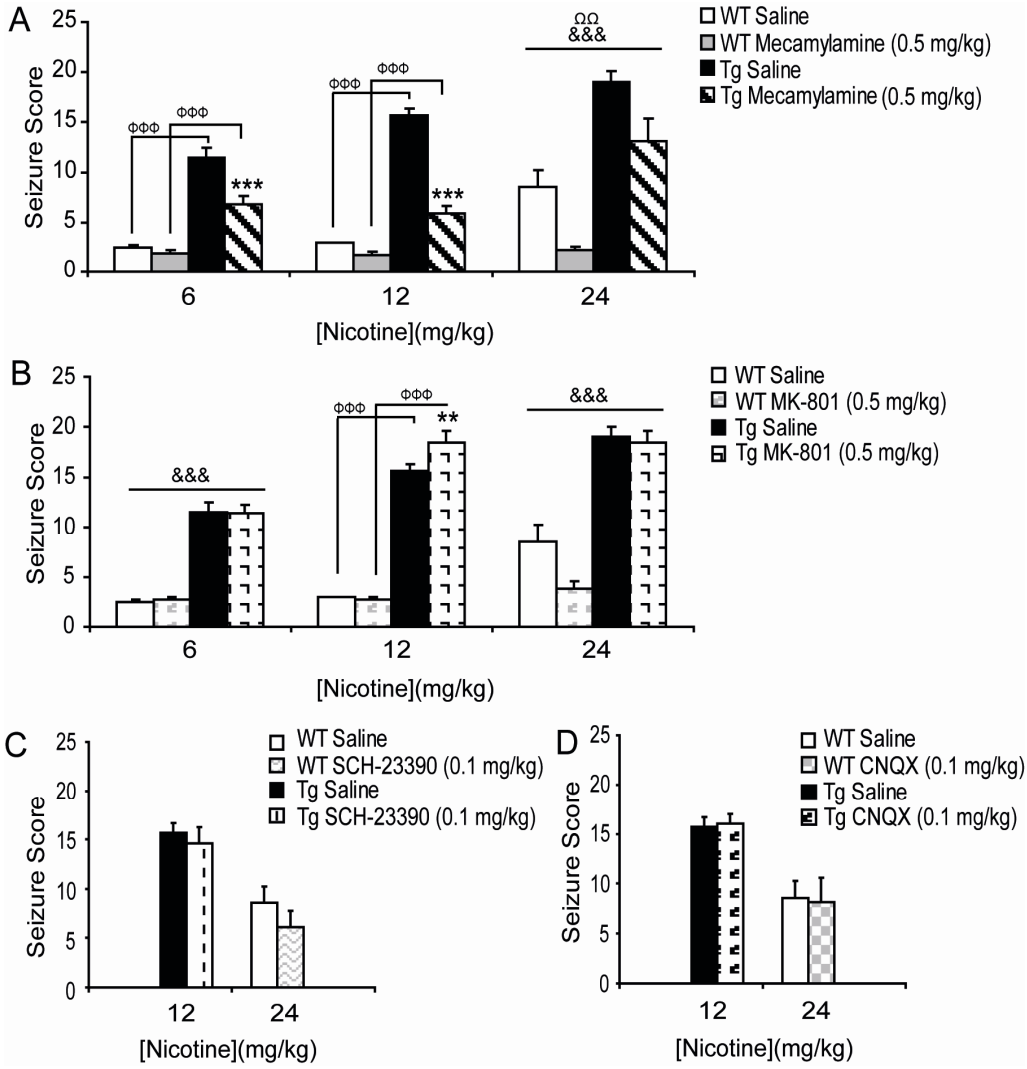


Figure 2. Cholinergic mechanisms involved in the increased sensitivity to nicotine-induced seizures of Tg*CHRNA5/A3/B4* mice. (A) Seizure score for nicotine (6, 12 and 24 mg/kg) in WT mice that received saline (open) or 0.5 mg/kg mecamylamine (gray) and Tg*CHRNA5/A3/B4* mice injected with saline (filled) or 0.5 mg/kg mecamylamine (striped). **(B)** Seizure score for nicotine (6, 12 and 24 mg/kg) in WT mice that received saline (open) or 0.5 mg/kg MK-801 (gray striped) and Tg*CHRNA5/A3/B4* mice injected with saline (filled) or 0.5 mg/kg MK-801 (black striped) 10 minutes before nicotine administration. **(C)** Seizure score for nicotine (12 and 24 mg/kg) in WT mice that received saline (open) or 0.1 mg/kg SCH-23390 (gray lined) and Tg*CHRNA5/A3/B4* mice injected with saline (filled) or 0.1 mg/kg SCH-23390 (black lined) 10 minutes before nicotine administration. **(D)** Seizure score for nicotine (12 mg/kg and 24 mg/kg) in WT mice that received saline (open) or 0.1 mg/kg CNQX (gray square) and Tg*CHRNA5/A3/B4* mice injected with saline (filled) or 0.1 mg/kg CNQX (black dot) 10 minutes before nicotine administration. N = 8-16 mice per group. Data are expressed as mean ± S.E.M. Two-way ANOVA treatment effect ΩΩ $p < 0.01$ and genotype effect &&& $p < 0.001$; Two-way ANOVA genotype x pre-treatment interaction ($p < 0.05$). Bonferroni *post hoc* comparisons between pre-treatment (** $p < 0.01$; *** $p < 0.001$) and between genotype (Φ $p < 0.05$; ΦΦ $p < 0.01$; ΦΦΦ $p < 0.001$).

Brain activation pattern upon nicotine-induced seizures: c-Fos expression analysis

Nicotine binding to nAChRs favours the increase of calcium inside the neuron, either directly (nAChR permeable to calcium) or indirectly (membrane depolarization that opens L-type calcium channels, VGCC), thus activating intracellular second messengers and initiating the expression of immediate early genes, such as c-Fos (Albuquerque et al., 1997; Damaj et al., 1999). c-Fos expression occurs rapidly, usually within few minutes, and the c-Fos protein can be detected between 90 and 120 minutes after the stimulus (Morgan et al., 1987). Substantial evidence suggests that the piriform and entorhinal cortices and the hippocampus are the major structures involved in limbic seizures induced by various stimuli (including nicotine) (Bastlund et al., 2005). Additionally, electrophysiological studies have indicated that nicotine-induced seizures originate in the hippocampus (Floris et al., 1964). 120 minutes after acute injection of either saline or nicotine (6 mg/kg) mice were sacrificed for c-Fos expression analysis in brain regions involved in seizures. Since this nicotine dose only produced seizures in Tg*CHRNA5/A3/B4* animals, a group of WT mice receiving a dose of 24 mg/kg nicotine was included, to allow comparison of brain activation pattern when nicotine-elicited clonic seizures in both genotypes. Two-way ANOVA between saline- and nicotine (6 mg/kg)-treated groups revealed a significant genotype x treatment interaction in the number of c-Fos positive nuclei per area (μm^2) in the stratum oriens (SO) [$F_{1,15} = 5.302$, $p < 0.05$] and stratum radiatum (SR) [$F_{1,15} = 17.677$, $p < 0.001$] of the dorsal CA1 subregion of the hippocampus (Fig. 3A and C). Bonferroni *post hoc* comparisons demonstrated that nicotine (6 mg/kg) significantly increased c-Fos expression in the CA1 SO ($p < 0.01$) and CA1 SR ($p < 0.001$) of Tg*CHRNA5/A3/B4* mice with respect to basal levels, but had no effect in WT, in which seizures were no observed. Other hippocampal strata were not activated by nicotine treatment (6 mg/kg) in either genotype (Fig. 3A and B). Interestingly, in WT mice, the dose of nicotine (24 mg/kg) eliciting seizures, only increased c-Fos expression in the granular cell layer (GCL) of the dentate gyrus (DG) ($p < 0.05$). The results showed a qualitatively different activation pattern when nicotine elicited seizures in WT and Tg*CHRNA5/A3/B4* animals and suggested that hypersensitivity to nicotine in Tg*CHRNA5/A3/B4* mice may be due to an increased activation of the CA1 SO and CA1 SR hippocampal subregions.

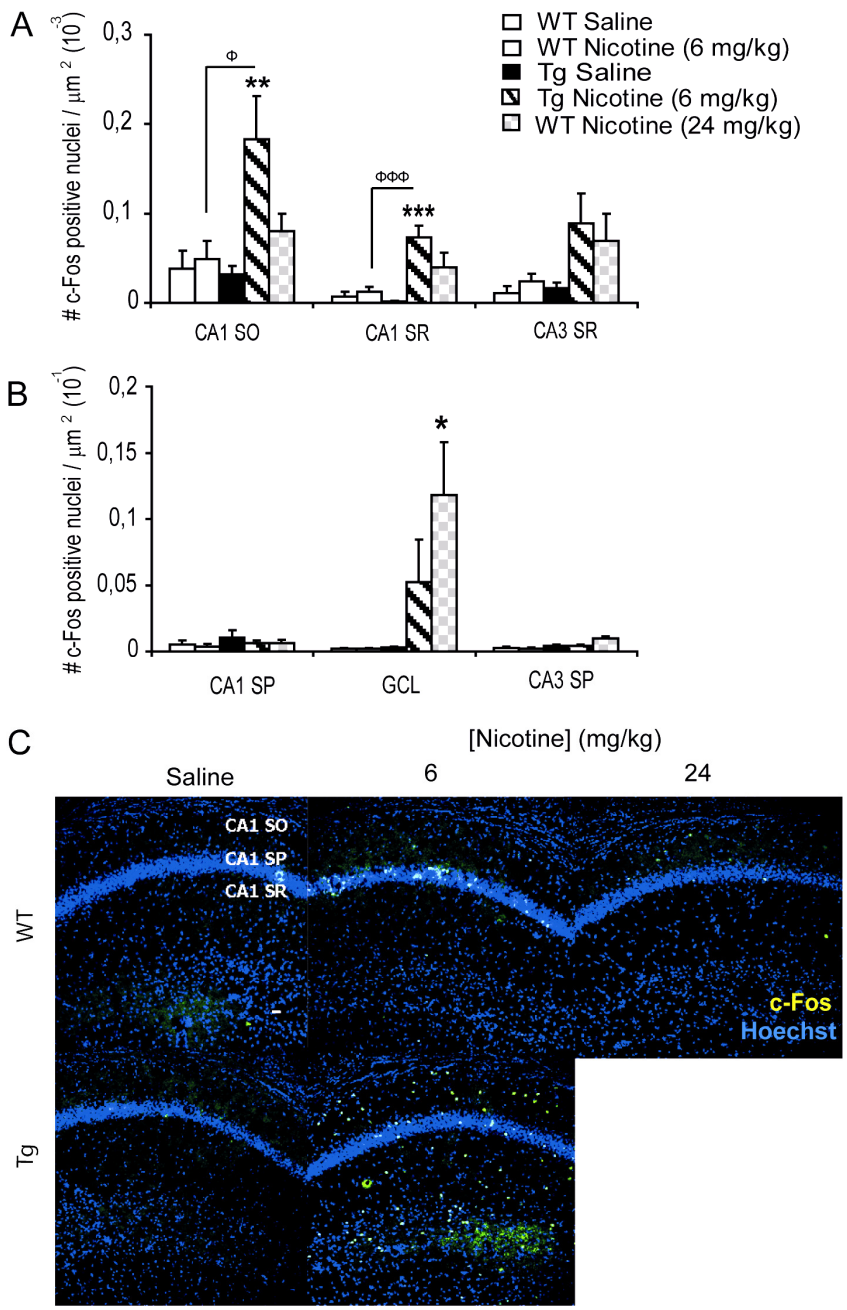


Figure 3. Nicotine induces differential activation of hippocampal subregions in Tg*CHRNA5/A3/B4* and WT mice. (A, B) c-Fos positive nuclei per area (μm^2) in mice that received an acute injection of saline (WT = open; Tg*CHRNA5/A3/B4* = filled) or nicotine (WT 6 mg/kg = gray; WT 24 mg/kg = gray squared; Tg*CHRNA5/A3/B4* 6 mg/kg = striped). **(C)** Photomicrograph illustrating c-Fos immunoreactive neurons in the CA1 subregion of the hippocampus. Slices are fluorescently labelled with c-Fos (green) and Hoechst (blue). Scale bar = 25 µm. SO: stratum oriens; SP: stratum pyramidale; SR: stratum radiatum; GCL: granular cell layer. N = 4 mice per group. Data are expressed as mean ± S.E.M. Two-way ANOVA genotype x treatment interaction ($p < 0.05$). Bonferroni *post hoc* comparisons between treatment (*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) and genotype (Φ $p < 0.05$; ΦΦΦ $p < 0.001$).

When the activation of the piriform cortex and lateral entorhinal cortex were examined, two-way ANOVA revealed a significant genotype x treatment interaction in the number of c-Fos positive nuclei in the piriform cortex [$F_{1,15} = 17.677$, $p < 0.001$] at 6 mg/kg nicotine (Fig. 4A and B). *Post hoc* comparisons showed that nicotine (6 mg/kg) significantly increased the expression of c-Fos in Tg*CHRNA5/A3/B4* mice ($p < 0.001$) but had no effect in their WT littermates. In the same region, a seizure-inducing nicotine dose (24 mg/kg) also increased c-Fos positive nuclei in nicotine- vs. saline-injected WT mice ($p < 0.001$). On the contrary, in the lateral entorhinal cortex, two-way ANOVA showed no significant effect of treatment or genotype x treatment interaction at the dose of nicotine of 6 mg/kg (Fig. 4A). However, in WT mice 24 mg/kg of nicotine significantly increased c-Fos expression in this region ($p < 0.01$).

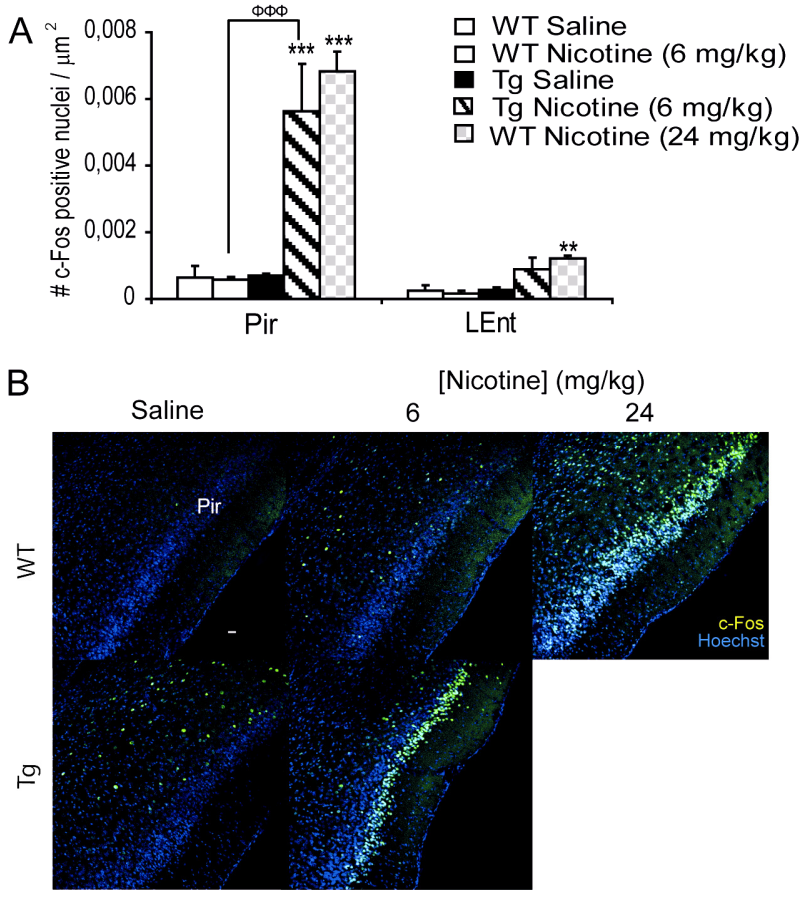


Figure 4. Differential activation of piriform and lateral entorhinal cortices in Tg*CHRNA5/A3/B4* upon nicotine administration. (A) c-Fos positive nuclei per area (μm^2) in mice treated with saline (WT = open; Tg*CHRNA5/A3/B4* = filled) or nicotine (WT 6 mg/kg = gray; WT 24 mg/kg = gray squared; Tg*CHRNA5/A3/B4* 6 mg/kg = striped). **(C)** Photomicrograph illustrating c-Fos immunoreactive neurons in the piriform cortex. Slices are fluorescently labelled with c-Fos (green) and Hoechst (blue). Scale bar =

25 µm. N = 4 mice per group. Pir = piriform cortex; LEnt = Lateral Entorhinal cortex. Data are expressed as mean ± S.E.M. Two-way ANOVA genotype x treatment interaction ($p < 0.05$). Bonferroni *post hoc* comparisons between treatment (** $p < 0.01$; *** $p < 0.001$) and between genotype (ΦΦΦ $p < 0.001$).

Finally, the involvement of other subregions such as the MHb, LHb, VTA and LC was examined. Two-way ANOVA demonstrated that 6 mg/kg nicotine significantly increased c-Fos expression in MHb of both WT and Tg*CHRNA5/A3/B4* mice [treatment effect, $F_{1,15} = 28.454$, $p < 0.001$] (Fig. 5A and B). In WT mice, 24 mg/kg of nicotine also increased c-Fos positive nuclei in the MHb ($p < 0.001$) (both lateral and medial parts) compared to saline-treated WT mice. Noticeably, the dose of 6 mg/kg activated the lateral cholinergic part of the MHb both in WT and Tg*CHRNA5/A3/B4* mice, immunolabelled against choline-acetyl transferase (ChAT, the enzyme responsible for the synthesis of acetylcholine) (Fig. 5 A and B).

In contrast, the LHb was not activated by any of the doses of nicotine tested. In the VTA and LC, two-way ANOVA revealed higher c-Fos expression upon nicotine (6 mg/kg) treatment [$F_{1,15} = 8.934$, $p < 0.05$; $F_{1,15} = 22.491$, $p < 0.001$, respectively], but there was no significant genotype x treatment interaction. Moreover, WT mice receiving an acute injection of 24 mg/kg nicotine showed a significant activation of the VTA and LC compared to saline-treated WT mice ($p < 0.01$, $p < 0.001$) (Fig.5A).

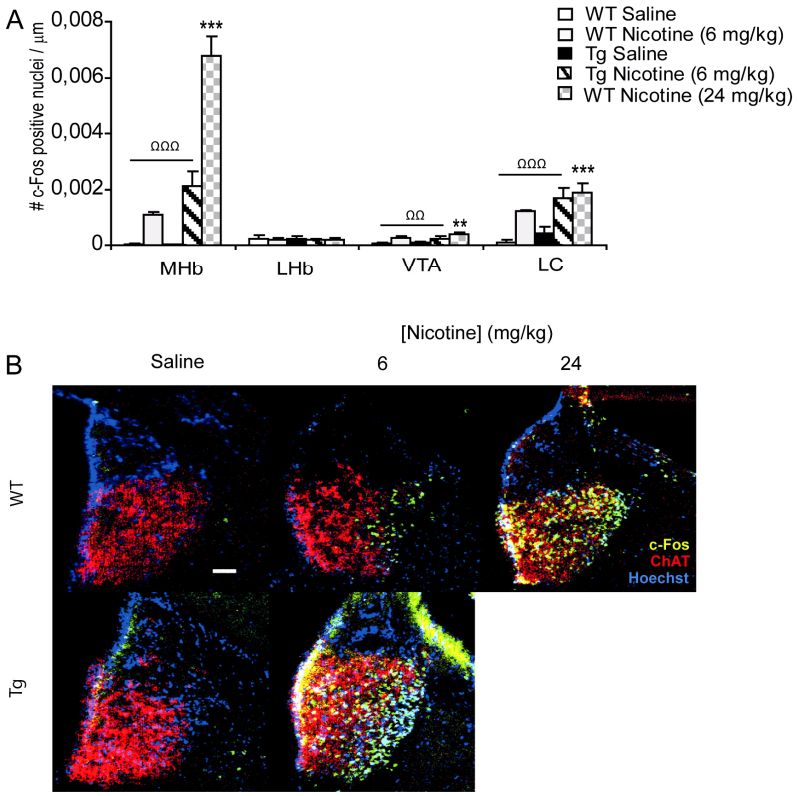


Figure 5. Tg*CHRNA5/A3/B4* and WT mice showed no differences in nicotine-induced activation of MHb, VTA, LHb or LC. (A) c-Fos positive nuclei per area (μm^2) in mice that received an acute injection of saline (WT = open; Tg*CHRNA5/A3/B4* = filled) or nicotine (WT 6 mg/kg = gray; WT 24 mg/kg = gray squared; Tg*CHRNA5/A3/B4* 6 mg/kg = striped). **(B)** Photomicrograph illustrating c-Fos immunoreactive neurons in the MHb. Slices are fluorescently labelled with c-Fos (green), ChAT (Red) and Hoechst (blue). Scale bar = 25 μm. N = 4 mice per group. Data are expressed as mean ± S.E.M. Two-way ANOVA treatment effect ΩΩ $p < 0.01$, ΩΩΩ $p < 0.001$. Bonferroni *post hoc* comparisons between treatment (** $p < 0.01$, *** $p < 0.001$).

b. Sensitivity to other pro-convulsive agents

We explored if overexpression of the cluster *CHRNA5/A3/B4* altered sensitivity to other pro-convulsive agents that also originate limbic seizures, such as glutamatergic agonists or GABAergic antagonists (Bastlund et al., 2005; Mandhane et al., 2007).

Experiment 1

NMDA, an agonist of the glutamatergic system that binds to NMDARs, was administered in a wide range of doses (110, 170, 190 or 210 mg/kg). Randomly assigned WT and Tg*CHNRA5/A3/B4* mice were injected with NMDA and examined for seizure manifestation along 30 minutes. Two-way ANOVA demonstrated no significant differences in seizure score between genotypes (Fig. 6A). Additionally, the percentage of Tg*CHNRA5/A3/B4* mice showing clonic/tonic seizures was similar to WT mice (Fig. 6B), suggesting that overexpression of the cluster *CHRNA5/A3/B4* does not modify sensitivity to NMDA.

Experiment 2

In another set of experiments the effects of inhibition of the GABAergic system were investigated using pentylenetetrazole (PTZ), a GABARs antagonist. WT and Tg*CHRNA5/A3/B4* mice received PTZ (50 mg/kg), and were evaluated for seizure manifestation during 30 min. Once more, two-way ANOVA demonstrated no significant differences between WT and Tg*CHRNA5/A3/B4* mice in seizure score (Fig. 6C). Moreover, the percentage of mice that displayed clonic/tonic seizures was equal between genotypes (Fig. 6D). However, when considering other seizure parameters, significant differences were observed between genotypes. For instance, the percentage

of Tg*CHRNA5/A3/B4* mice that elicited Straub tail seizure were 75 % compared to the 37,5% presented by their WT littermates. About 75% of Tg*CHRNA5/A3/B4* mice exhibited myoclonic seizures vs. 25% of WT mice. Additionally, the latency to elicit myoclonic seizure was significantly lower in Tg*CHRNA5/A3/B4* animals [Student's T test, $t_{1,6} = 4.333$, $p < 0.01$] (Fig. 6E).

The present results suggested that Tg*CHRNA5/A3/B4* mice might have alterations in GABAergic system, resulting in a slightly increased sensitivity to pro-convulsive agents that block the inhibitory transmission.

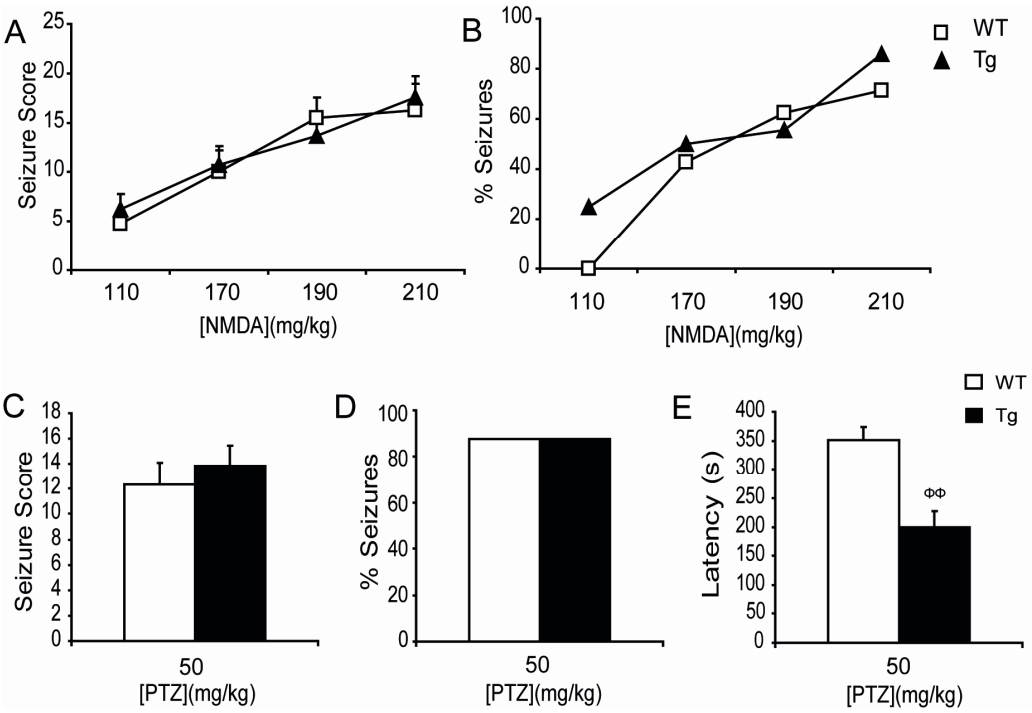


Figure 6. Tg*CHRNA5/A3/B4* mice show normal sensitivity to NMDA-induced seizures but slightly increased sensitivity to PTZ-induced seizures. (A) Seizure score and **(B)** percentage of mice exhibiting clonic/tonic seizures in WT (open) and Tg*CHRNA5/A3/B4* (filled) mice that received an acute injection of NMDA in a wide range of doses (110, 170, 190 and 210 mg/kg). N = 7-10 mice per group. **(C)** Seizure score, **(D)** percentage of mice exhibiting clonic/tonic seizures and **(E)** latency (s) to present myoclonic seizures in WT (open) and Tg*CHRNA5/A3/B4* (filled) mice that received an acute injection of PTZ (50 mg/kg). N = 8 mice per group. Data are expressed as mean ± S.E.M. Student's T test (ΦΦ $p < 0.01$).

4.2. CHARACTERIZATION OF THE COGNITIVE PROFILE IN Tg*CHRNA5/A3/B4* MICE

The probability to develop drug addiction is substantially different among individuals. Indeed, the co-morbidity between nicotine addiction with mental illness is particularly high for psychiatric conditions, suggesting that alterations in specific neurological traits predispose an individual to develop addiction to nicotine (Reviewed in (Janet Audrain-McGovern, 2009)). The second part of the Thesis focused on investigating how the overexpression of the cluster *CHRNA5/A3/B4* affected different aspects of cognitive performance associated to nicotine addiction and how nicotine administration either acutely or chronically modulated these cognitive tasks.

4.2.1. The novel object discrimination task

Quantitative or qualitative alterations in attention/working memory may be one trait predisposing an individual to develop nicotine addiction. The novel object discrimination task examined whether mice had the ability to discriminate between a familiar and a novel object, which may be considered to be an index of working memory/attention. The task takes advantage of the natural tendency of rodents to explore novelty. In the novel object discrimination task, mice are presented with two identical objects and allowed to explore them for 10 min. After 1h delay, animals are presented again with two objects for 5 min, one of which is familiar and the other a new one. The difference in the time spent exploring the novel object respect to the familiar one corrected by the total time of exploration is considered as discrimination index (Dere et al., 2007).

Experiment 1

Novel object discrimination was examined in WT and Tg*CHRNA5/A3/B4* mice under basal conditions. Student's T test analysis demonstrated no significant differences between genotypes regarding total exploration time during the familiarization session (Fig. 7A). Moreover, there were no signs of anxiety-like behaviour or changes in locomotor activity in Tg*CHRNA5/A3/B4* mice, since the time spent in centre and periphery and the total distance travelled during the familiarization session were equal

compared to their WT littermates (Fig. 7B,C). During the test session, Tg*CHRNA5/A3/B4* animals presented a significant deficit in novel object discrimination [$t_{1,20}$ = 2.302, $p < 0.05$] (Fig. 7D), suggesting that overexpression of the cluster *CHRNA5/A3/B3* impaired the capacity to discriminate novelty compared to familiarity. This deficit was not attributable to changes in total time of exploration, time in centre and periphery nor total distance travelled (data not shown).

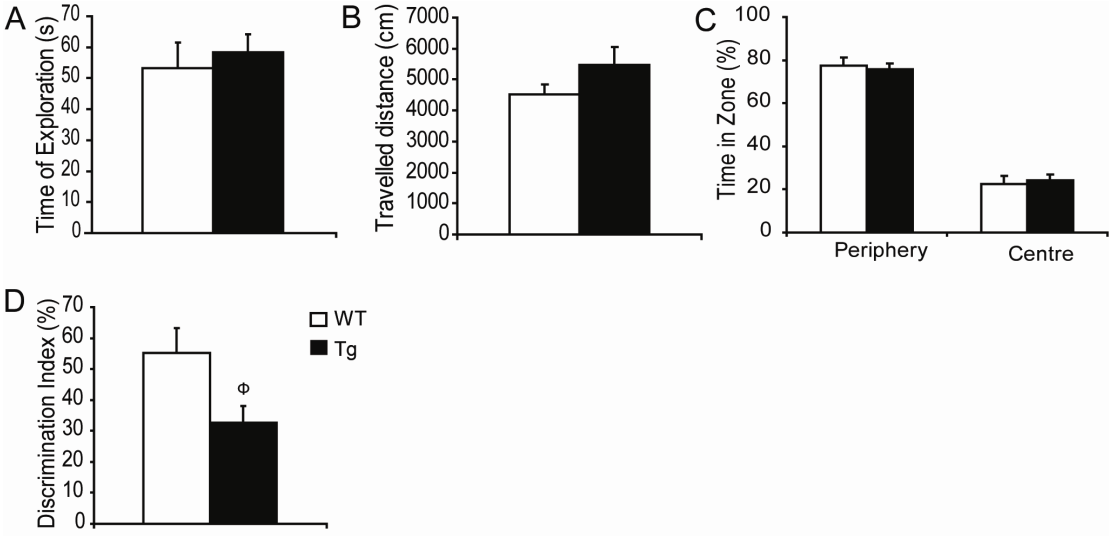


Figure 7. Tg*CHRNA5/A3/B4* mice show a deficit in novel object discrimination. (A) Total time of exploration (s), **(B)** total distance travelled (cm) and **(C)** percentage of time in periphery and centre during the familiarization session, in WT (open) and Tg*CHRNA5/A3/B4* (filled) mice under basal situation. **(D)** Novel object discrimination index during the test session (time exploring the novel object – time exploring the familiar object) /total time of exploration*100. N = 10-12 mice per group. Data are expressed as mean ± S.E.M. Student's T test Φ $p < 0.05$.

Experiment 2

With the aim of investigating the effects of acute nicotine administration on novel object discrimination, mice received an acute injection of either saline or nicotine (0.1 mg/kg) 5 minutes before the familiarization (learning) session. Because of the increased sensitivity to nicotine in Tg*CHRNA5/A3/B4* mice, the dose of nicotine of 0.1 mg/kg was selected according to previous results in the laboratory demonstrating that it does not alter normal behaviour in these mice (i.e. exploratory activity or grooming episodes) (unpublished data). Furthermore, a dose of 0.09 mg/kg, produces plasma nicotine levels in mice similar to those in smokers (Davis et al., 2006). What is more, systemic 0.1 mg/kg nicotine is enough to improve discrimination memory in C57BL mice

(Melichercik et al., 2012). Two-way ANOVA indicated that nicotine did not alter anxiety-like behaviour or locomotor activity during the familiarization session, with neither genotype or treatment effect nor genotype x treatment interaction in total time of exploration, time in centre and periphery, nor total distance travelled (Fig. 8A, B and C). However, there was a significant genotype x treatment interaction on discrimination index [$F_{1,56}$ = 4.393, $p < 0.05$]. Bonferroni *post hoc* comparisons between individual groups demonstrated that acute nicotine administration was able to restore Tg*CHRNA5/A3/B4* mice deficits ($p < 0.05$) (Fig. 8D). In contrast, nicotine did not modify novelty discrimination in WT animals. The present data indicated that acute nicotine was able to restore the novel object discrimination deficit observed in mice overexpressing the cluster *CHRNA5/A3/B4*.

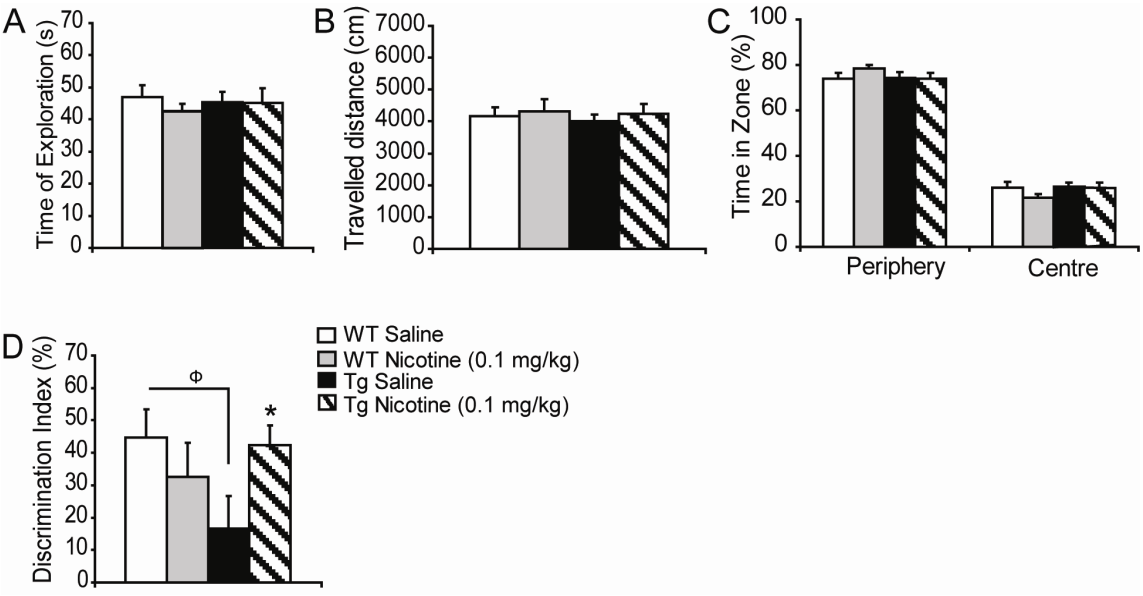


Figure 8. Acute nicotine administration restores the novel object discrimination deficit observed in Tg*CHRNA5/A3/B4* mice. (A) Total exploration time (s), **(B)** total distance travelled (cm) and **(C)** percentage of time in periphery and centre during the familiarization session in WT mice that received either saline (open) or 0.1 mg/kg nicotine (gray) and Tg*CHRNA5/A3/B4* mice treated with saline (filled) or 0.1 mg/kg nicotine (striped) 5 minutes before the familiarization session. **(D)** Novel object discrimination index during the test session (time exploring the novel object – time exploring the familiar object) /total time of exploration*100. N = 14 mice per group. Data are expressed as mean ± S.E.M. Two-way ANOVA genotype x treatment interaction ($p < 0.05$). Bonferroni *post hoc* comparisons between treatment (* $p < 0.05$) and between genotype (Φ $p < 0.05$).

Experiment 3

In a separate set of animals we studied whether chronic nicotine infusion modified the reduced object discrimination observed due to overexpression of the cluster *CHRNA5/A3/B4*. Mice were implanted with osmotic minipumps delivering either saline or nicotine (10 mg/kg/d) and after 5 days of chronic treatment they were tested in the novel object discrimination task. In rats, osmotic minipumps providing constant, slow delivery of nicotine of 2-4 mg/kg/d achieves plasma nicotine concentrations of 20-50 ng/ml (Barik and Wonnacott, 2006), similar to that reached in current smokers assuming regular cigarette consumption during the day (Gourlay and Benowitz, 1997). Thus, the dose of nicotine selected was further increased because of the fact that the rate of metabolism in mice is much faster than in rats. Additionally, the dose of nicotine selected was sufficient to induce nicotine withdrawal in mice (Castane et al., 2002) and it would be interesting to check whether the same dose was able to modify cognitive processes. In chronically implanted mice, two-way ANOVA revealed a significant nicotine effect on total distance travelled [$F_{1,44} = 4.34$, $p < 0.05$] (Fig. 9 B,C) that was reduced in both genotypes during the familiarization session, without affecting total exploration time (Fig. 9A). During the test session, there was a significant genotype x treatment interaction on the discrimination index [$F_{1,44} = 4.094$, $p < 0.05$]. Bonferroni *post hoc* comparisons again showed that saline-treated Tg*CHRNA5/A3/B4* mice presented reduced novel object discrimination in comparison to saline-treated WT ($p < 0.01$) (Fig. 9D) as observed in non-treated conditions (see above). Interestingly, chronic nicotine administration tended to restore the deficit observed in Tg*CHRNA5/A3/B4* mice, although restoration did not reach statistical significance ($p = 0.071$). Again, nicotine did not modify novel object discrimination in WT animals.

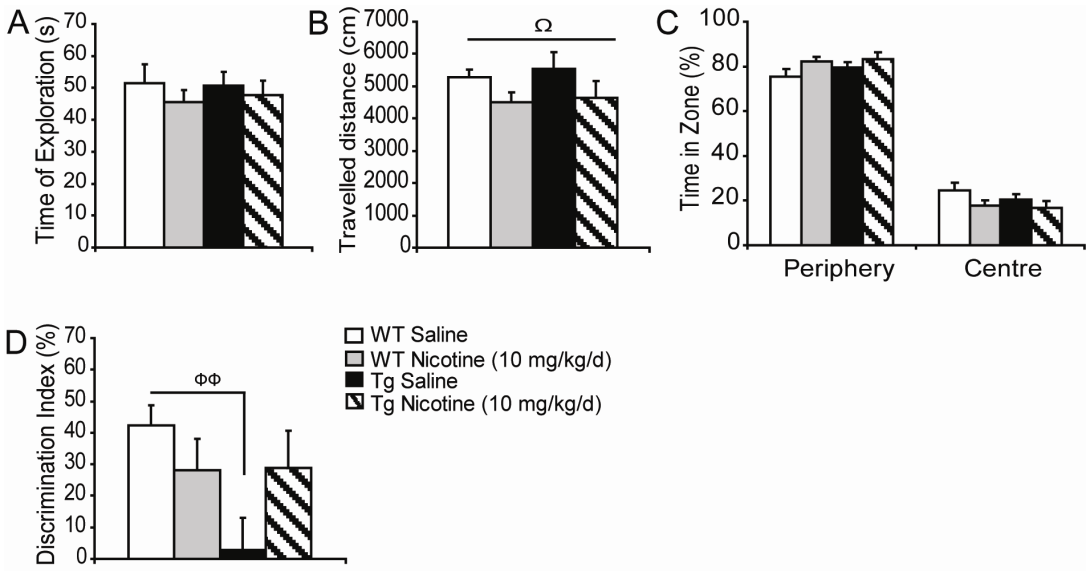


Figure 9. Chronic nicotine treatment partially restores the novel object discrimination deficit of Tg*CHRNA5/A3/B4* mice. (A) Total time of exploration (s), **(B)** total distance travelled (cm) and **(C)** percentage of time in periphery and centre during the familiarization session, in WT mice that received either saline (open) or 10 mg/kg/d nicotine (gray) and Tg*CHRNA5/A3/B4* mice treated with saline (filled) or 10 mg /kg/d nicotine (striped) for 5 days before the familiarization session. **(D)** Novel object discrimination index during the test session (time exploring the novel object – time exploring the familiar object)/total time of exploration*100. N = 10-12 mice per group. Data are expressed as mean ± S.E.M. Two-way ANOVA genotype x treatment interaction ($p < 0.05$). Two way ANOVA treatment effect (Ω $p < 0.05$); Bonferroni *post hoc* comparisons between genotype (ΦΦ $p < 0.01$).

4.2.2. The recurrent behaviour task

Recurrent exploration (see Methods section) was used to further investigate working memory and perseverant behaviour in WT and Tg*CHRNA5/A3/B4* mice. Animals were presented with two identical objects and the frequency of revisiting the same object one, two, three or more than 3 consecutive times was analyzed. Student's T test demonstrated non-significant differences between genotypes when evaluating low number of revisits to the same object (one, two and three consecutive times exploring the same object). However, a significant difference between WT and Tg*CHRNA5/A3/B4* mice was revealed when analyzing the highest number of revisits [> 3; $t_{(1,23)} = -2.603$; $p < 0.05$] (Fig. 10), suggesting that Tg*CHRNA5/A3/B4* mice have a less efficient working memory function and/or increased perseverant behaviour compared to their WT littermates.

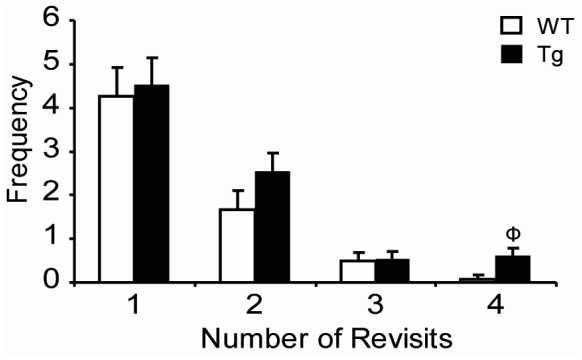


Figure 10. Tg*CHRNA5/A3/B4* mice show increased recurrent behaviour. Frequency to revisit the same object one, two, three or more consecutive times during a 10 minutes session, in WT (open) and Tg*CHRNA5/A3/B4* (filled) mice. N = 12-13 mice per group. Data are expressed as mean ± S.E.M. Student's T test Φ $p < 0.05$.

4.2.3. The passive avoidance paradigm

Deficits in recent memory and behavioural inhibition are predisposing traits for development of nicotine addiction. The passive avoidance test allows investigating these two cognitive abilities. On training day mice are placed in a small platform and when stepped down they received a foot shock. On the test day, after 24 hours or 7 days, mice had to remember the foot shock and inhibit stepping down from the platform.

Experiment 1

Avoidance learning was examined in WT and Tg*CHRNA5/A3/B4* mice. Student's T test analysis showed no significant genotype effect on the training day. However, 24 hours later, on test day, the step down latency was significantly lower in Tg*CHRNA5/A3/B4* animals [$t_{1,46} = 4.077$, $p < 0.05$] compared to their WT littermates (Fig. 11A), suggesting impairment in avoidance learning when the *CHRNA5/A3/B4* cluster is overexpressed.

Experiment 2

The effects of acute nicotine administration on avoidance learning were studied in mice receiving an acute injection of either saline or nicotine (0.1 mg/kg) 5 minutes prior to the training session. No significant effect of genotype, treatment, or genotype x

treatment interaction was observed on the training day. Nonetheless, on the testing day two-way ANOVA yielded a significant genotype x treatment interaction on step down latency [$F_{1,75}$ = 4.337, $p < 0.05$]. Bonferroni *post hoc* comparisons confirmed that saline-treated Tg*CHRNA5/A3/B4* animals stepped down earlier than their saline-treated WT littermates ($p < 0.05$) (Fig. 11B). One week after the training session, two-way ANOVA revealed a significant genotype x treatment interaction [$F_{1,75}$ = 7.474, $p < 0.01$]. Saline-treated Tg*CHRNA5/A3/B4* mice showed a reduced step down latency compared to WT (Bonferroni *post hoc* comparisons; $p < 0.05$). Nicotine had no significant effect in Tg*CHRNA5/A3/B4* animals, suggesting that although overexpression of the cluster reduced avoidance learning at short (24 hours) and long (7 days) delays after training, acute nicotine administration was not able to restore this phenotype. However, WT mice that received nicotine on training day stepped down earlier as compared to their saline-injected counterparts (Bonferroni *post hoc* comparisons; $p < 0.05$).

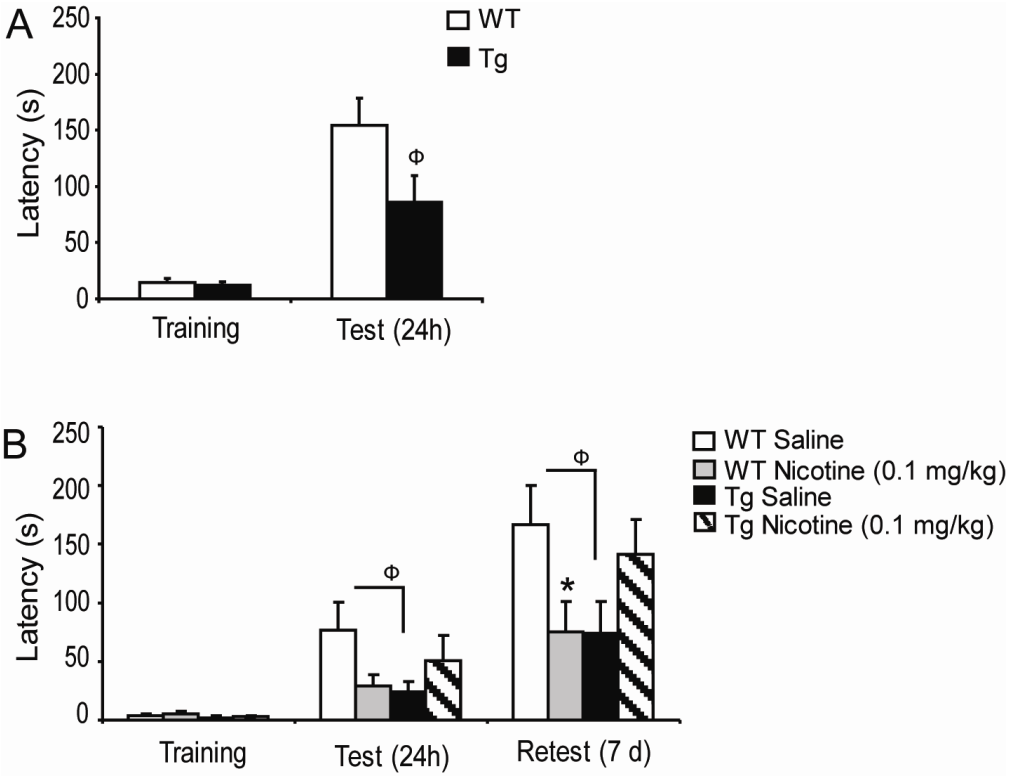


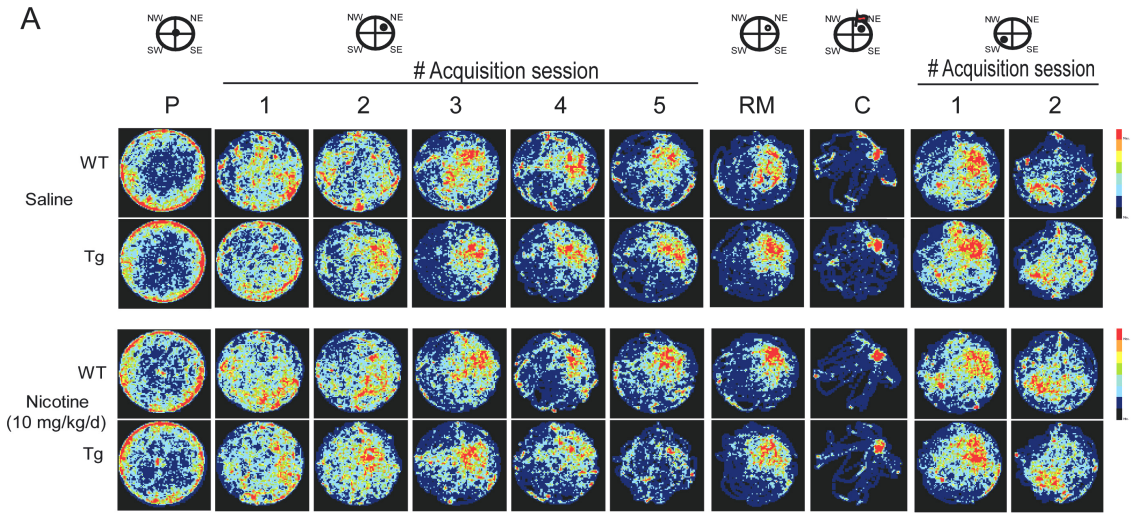
Figure 11. Tg*CHRNA5/A3/B4* mice show a deficit in avoidance learning that is not restored by acute nicotine (0.1 mg/kg) administration. **(A)** Latency (s) to step down from the platform in the passive avoidance task during the training and test (24 hours delay) sessions in WT (open) and Tg*CHRNA5/A3/B4* (filled) mice. N = 22-25 mice per group. **(B)** Latency (s) to step down off from the

platform in the passive avoidance task during the training, test (24 hours) and retest (7 days) sessions in WT mice that received either saline (open) or 0.1 mg/kg nicotine (gray) and Tg*CHRNA5/A3/B4* mice treated with saline (filled) or nicotine (0.1 mg/kg)(striped) 5 minutes before the training session. N = 17-21 mice per group. Data are expressed as mean ± S.E.M. Two-way ANOVA genotype x treatment interaction ($p < 0.05$). Student's T test Φ $p < 0.05$. Bonferroni *post hoc* comparisons between treatment (* $p < 0.05$) and between genotype (Φ $p < 0.05$).

4.2.4. The Morris Water Maze test

The Morris Water Maze test (MWM) is a suited model for studying goal-directed behaviours and cognitive flexibility. The MWM test was performed in WT and Tg*CHRNA5/A3/B4* mice implanted with osmotic minipumps delivering chronically either saline or nicotine (10 mg/kg/d). We did not explore the effects of acute nicotine administration since the MWM test requires several days of acquisition. Studies examining the effects of acute nicotine on acquisition of this task require nicotine administration over multiple days. This raises the question as to whether such studies are examining the effects of acute or chronic nicotine on acquisition (Giniatullin et al., 2005). The test started 24 hours after minipump implantation with a pre-training session in which animals learn the procedural aspect of the task. Two-way ANOVA revealed that nicotine improved procedural learning in both genotypes [$F_{1,56} = 6.966$, $p < 0.05$] (Fig. 12B). Then, animals are given a series of acquisition sessions, in which the platform is hidden on the North-East (NE) quadrant of the pool. Mice must use visuospatial cues to create an allocentric map that allows them to be oriented within the space and to reach the goal intended (Morris, 1984). Repeated measures ANOVA showed similar visuospatial learning in all groups along the acquisition session, as reflected by the decrease in escape latency across sessions (Fig. 12B). However, when the time spent in the trained quadrant (NE) along the acquisition sessions (considered to be indicative of learning) was analyzed, repeated measures ANOVA revealed a significant genotype x treatment interaction [$F_{1,50} = 4.085$, $p < 0.05$] (Fig. 12C). *Post hoc* comparisons between groups revealed that saline-treated Tg*CHRNA5/A3/B4* mice spent more time in the trained quadrant than saline-treated WT littermates ($p = 0.06$; $p < 0.01$; $p < 0.01$; $p = 0.096$; $p = 0.491$; acquisition sessions 1, 2, 3, 4 and 5, respectively) indicating more efficient visuospatial learning in animals overexpressing the cluster *CHRNA5/A3/B4*. Interestingly, nicotine treatment significantly reduced the time spent in

the trained quadrant only in Tg*CHRNA5/A3/B4* mice ($p = 0.926$; $p < 0.05$; $p < 0.05$; $p = 0.102$; $p = 0.997$; acquisition sessions 1, 2, 3, 4 and 5, respectively) but had no effect in their WT littermates. These results were not due to genotype differences in locomotor activity or motivation since two-way ANOVA did not show significant differences among groups in latency or swimming speed during the cued session (Fig. 12B). In the probe session when the platform was removed and reference memory was examined, no significant differences in time spent at each quadrant among the four groups (Fig. 12D), nor genotype x treatment interaction in the number of entries to the trained platform zone were observed [$F_{1,56} = 2.857$, $p = 0.097$] (Fig. 12E). However, saline-treated Tg*CHRNA5/A3/B4* mice tended to enter more times into the trained platform zone compared to the saline-treated WT littermates. Chronic nicotine treatment tended to compensate this behaviour only in mice overexpressing the cluster *CHRNA5/A3/B4* but had no effect in control mice. Finally, when the platform was situated on the opposite quadrant of the pool (South-West, SW) to analyse cognitive flexibility, repeated measures ANOVA indicated an overall learning effect across the two reversal sessions in all groups but no significant genotype, treatment effect, or genotype x treatment interaction in latency to reach the new position of the platform (Fig. 12F). When analyzing the percentage of time that mice spent in the old trained quadrant (NE) vs. the new target quadrant (SW) during the two reversal sessions ANOVA repeated measures revealed that all groups significantly decreased the time spent in the old target quadrant and increased the time spent in the new trained quadrant (Fig. 12G). Chronic nicotine administration had no effect in cognitive flexibility. All groups' trajectories across the different sessions of the MWM are illustrated in Fig. 12A.



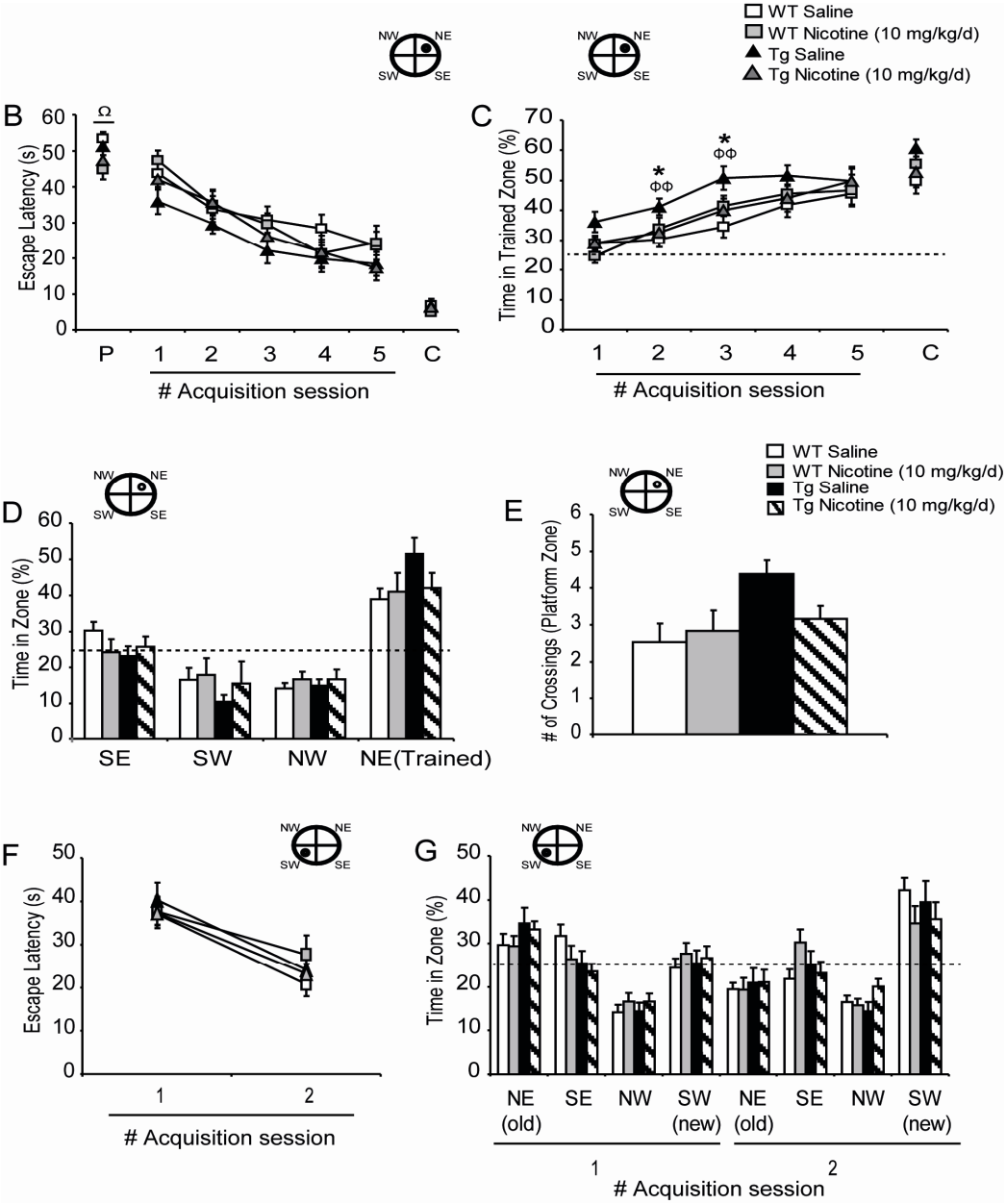


Figure 12. Visuospatial learning and reference memory in WT and Tg*CHRNA5/A3/B4* mice under basal situation and upon chronic nicotine administration. (A) Scatter plot illustrating the animals' trajectories across the different sessions. **(B)** Escape latency (s) during the pre-training, acquisition and cued sessions, in mice implanted with osmotic minipumps delivering either saline or 10 mg/kg/d nicotine 24 hours before the pre-training session (WT saline = open squares; Tg*CHRNA5/A3/B4* saline = filled triangles; WT nicotine = gray squares; Tg*CHRNA5/A3/B4* nicotine = gray triangles). **(C)** Percentage of time in the trained quadrant during the acquisition and cued sessions. **(D)** Percentage of time in the four quadrants of the pool (WT saline = open; Tg*CHRNA5/A3/B4* saline = filled; WT nicotine = gray; Tg*CHRNA5/A3/B4* nicotine = striped) and **(E)** number of crossings through the target zone during the probe session. **(F)** Escape latency (s) and **(G)** percentage of time in the four quadrants of the pool during the two reversal sessions. N = 13-15 mice per group. Data are expressed as mean ± S.E.M. Two-way ANOVA treatment effect (Ω $p < 0.05$). Two-way ANOVA genotype x treatment interaction ($p < 0.05$). Bonferroni *post hoc* comparisons between treatment (* $p < 0.05$) and between genotype (ΦΦ $p < 0.01$).

4.2.5. The pure contextual fear conditioning paradigm

With repeated nicotine exposure contextual factors become associated to the reinforcing properties of the drug so that the exposure to such conditioned stimuli can eventually activate brain regions involved in reinforcement and induce cravings (Volkow et al., 2008). The contextual fear conditioning is suitable to investigate memories for contextual information, since it addresses the ability to create associations between a neutral conditioned stimulus (CS) (i.e. the context) and an unconditioned stimulus (US) (i.e. a foot shock). We used the pure-contextual fear conditioning paradigm in which the US represents exclusively the context of the conditioned chamber. Commonly, 24h after conditioning, the strength of the contextual association is tested by returning the conditioned animal to the training chamber and scoring freezing, a behavioural measure of fear (Gould, 2006). Memory for contextual associations was evaluated in WT and Tg*CHRNA5/A3/B4* mice that received either saline or nicotine (10 mg/kg/d) during 5 days. Two-way ANOVA revealed similar basal freezing levels among the four groups (Fig 13A). Repeated measures ANOVA indicated a significant genotype x treatment interaction in acquisition of freezing behaviour [$F_{1,49}$ = 3.317, $p < 0.1$] (Fig. 13A). Bonferrroni *post hoc* comparisons demonstrated no genotype differences between saline-treated groups. However, nicotine-treated Tg*CHRNA5/A3/B4* mice showed significantly less freezing time in the first ($p < 0.05$) and second ($p < 0.05$) shock administration compared to nicotine-treated WT animals. In the test session 24 hour after, two-way ANOVA did not show statistical differences between genotypes, treatment, nor genotype x treatment interaction regarding the freezing response (Fig. 13B). The present data suggests that overexpression of the cluster *CHRNA5/A3/B4* does not affect memories for contextual associations and chronic nicotine administration is not able to modulate this type of memories. However, nicotine administration had opposite genotype effects on acquisition learning.

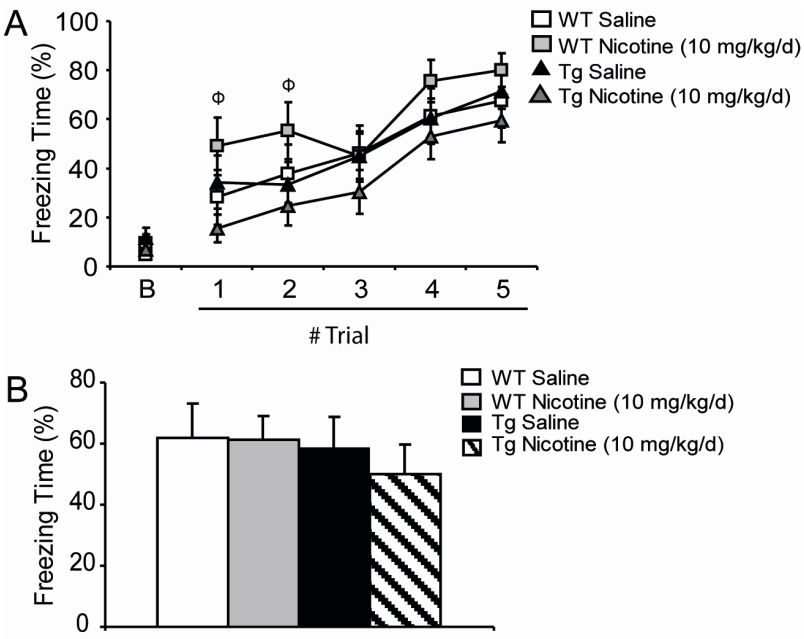


Figure 13. Tg*CHRNA5/A3/B4* mice show normal pure contextual memory and chronic nicotine had opposite genotype effects on acquisition learning. **(A)** Percentage of time freezing in basal situation and during 15 seconds after receiving each of the five foot shock presented, during the acquisition session, in WT mice that received either saline (open) or 10 mg/kg/d nicotine (gray) and Tg*CHRNA5/A3/B4* mice treated with saline (filled) or nicotine (10 mg/kg/d) (striped) during 5 days. **(B)** Percentage of time freezing in the 2 minutes session on the test day (24h delay). N = 11-16 mice per group. Data are expressed as mean ± S.E.M. ANOVA repeated measures genotype x treatment interaction ($p < 0.05$). Bonferroni *post hoc* comparisons between genotype (Φ $p < 0.05$).

In summary, the effects of overexpression of the cluster *CHRNA5/A3/B4* on cognitive performance profoundly depended on the task examined. Furthermore, acute or chronic administration of nicotine had different effects depending on the genotype. These results may indicate the multitude of brain regions, neurotransmitter systems and nAChRs receptors involved in each behavioural paradigm. Table 2 summarizes the data obtained from all behavioural tests performed and the responses to nicotine treatment, either acutely or chronically.

Test	Cognitive domain addressed	Genotype	Treatment	Behavioural response
Object discrimination	Working memory/ Attention	WT	Acute Nicotine	
			Chronic Nicotine	
		Tg	Basal	
			Acute Nicotine	
			Chronic Nicotine	
Passive Avoidance	Behavioural Inhibition	WT	Acute Nicotine	
			Chronic Nicotine	nd
		Tg	Basal	
			Acute Nicotine	
			Chronic Nicotine	nd
Morris Water Maze	Visuospatial Learning/ Cognitive flexibility	WT	Acute Nicotine	nd
			Chronic Nicotine	
		Tg	Basal	
			Acute Nicotine	nd
			Chronic Nicotine	
Pure Contextual Fear Conditioning	Contextual memory	WT	Acute Nicotine	nd
			Chronic Nicotine	
		Tg	Basal	
			Acute Nicotine	nd
			Chronic Nicotine	

Table 2. Summary of each behavioural paradigm examined, the behavioural response of Tg*CHRNA5/A3/B4* mice compared to their WT littermates in basal situation and the effects of acute and chronic nicotine treatment. Gray = No effect; Green = Positive effect; Red = Deleterious effect; nd = not determined.

4.3. CHARACTERIZATION OF THE HIPPOCAMPUS IN Tg*CHRNA5/A3/B4* MICE

The hippocampus has been shown to be involved both in reward and learning processes and may influence development of addiction. Interestingly, previous results indicated that Tg*CHRNA5/A3/B4* mice had increased α3β4* nAChRs in the hippocampus, principally in the CA1 subregion (Gallego et al., 2011). We studied the morphological characteristics and functional connectivity of the hippocampus of Tg*CHRNA5/A3/B4* mice and its response to nicotine. Concretely, structural complexity and dendritic spine density of hippocampal pyramidal neurons were evaluated. Changes in these two parameters are some of the neuroadaptations that occur after nicotine administration and are important for the acquisition of maladaptive behaviours (Dietz et

al., 2009). The study was performed in the hippocampus from WT and Tg*CHRNA5/A3/B4* mice and in hippocampal primary cultures in which the impact of nicotine of structural plasticity could be analyzed in detail.

4.3.1. Morphological study of the hippocampus

To analyze the morphological parameters of the hippocampus Tg*CHRNA5/A3/B4* male mice were bred with *Thy1-YFP* heterozygous female mice to obtained WT and Tg*CHRNA5/A3/B4* mice expressing *YFP* in subsets of pyramidal neurons in the brain. This genetic tool allowed examining the length and number of neuronal somas in the different strata of the hippocampus. The morphometric study was performed in WT and Tg*CHRNA5/A3/B4* mice implanted with osmotic minipumps delivering either saline or nicotine (10 mg/kg/d) for 7 days. Two-way ANOVA analysis yielded a significant genotype x treatment interaction when evaluating the length of the stratum pyramidale in the dorsal CA1 subregion of the hippocampus [$F_{1,19} = 8.003$, $p < 0.05$] (Fig. 14A, C). Bonferroni *post hoc* comparisons demonstrated no significant differences between the nicotine-treated groups, but while chronic nicotine treatment tended to increase the length of CA1 stratum pyramidale layer in WT mice ($p = 0.062$) it slightly reduced the length of the same area in Tg*CHRNA5/A3/B4* animals ($p = 0.064$). As a result, nicotine-treated Tg*CHRNA5/A3/B4* mice showed a significant reduced length of the CA1 pyramidale layer compared to their nicotine-treated WT littermates ($p < 0.01$). No significant differences were observed in the length of other strata of the hippocampus (CA1 oriens, CA1 radiatum, lacunosum moleculare, moleculare or granular cell layer). Two-way ANOVA revealed that the differences observed were not due to changed number of neuronal somas in the CA1 stratum pyramidal layer as shown in Fig. 14B. Interestingly, chronic nicotine treatment altered the width of the pyramidal layer within the CA1 subregion of the hippocampus and this effect was totally opposed in mice overexpressing the cluster *CHRNA5/A3/B4*.

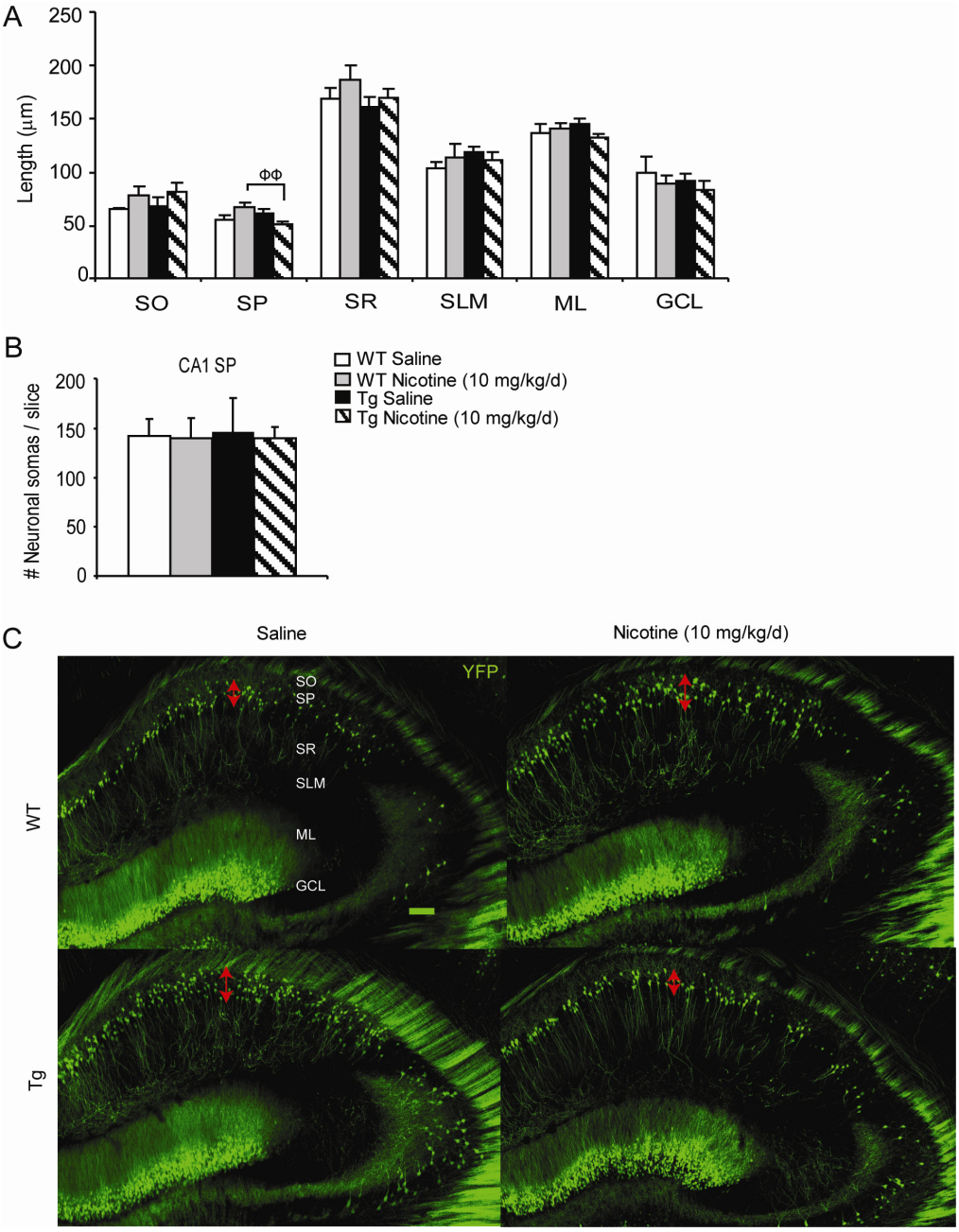


Figure 14. Effects of chronic nicotine (10 mg/kg/d, 7 days) on CA1 pyramidal neurons. **(A)** Length (µm) of the different strata of the hippocampal CA1 subregion in WT mice that received either saline (open) or nicotine (10 mg/kg/d)(gray) and Tg*CHRNA5/A3/B4* mice treated with saline (filled) or nicotine (10 mg/kg/d)(striped) for 7 days. **(B)** Number of neuronal somas per slice in the pyramidal CA1 subregion of the hippocampus. **(C)** Photomicrograph illustrating the hippocampus of *Thy1*-YFP mice from the four groups. Red arrowhead lines indicate differences in CA1 stratum pyramidale length among groups. Scale bar = 100 µm. SO: stratum oriens; SP: stratum pyramidale; SR: stratum radiatum; SLM: stratum lacunosum moleculare; ML: molecular layer; GCL: granular cell layer. N = 4 - 6 mice per group. Data are expressed as mean ± S.E.M. Two-way ANOVA genotype x treatment interaction ($p < 0.05$). Bonferroni *post hoc* comparisons between genotype (ΦΦ $p < 0.01$).

4.3.2. Structural complexity of pyramidal neurons

Neuronal complexity is profoundly influenced by neuronal activity and is fundamental in receiving, processing, and integrating inputs from synaptic partners. We used hippocampal primary cultures obtained from WT and Tg*CHRNA5/A3/B4* mice at embryonic day (E) 17.5-18.5, and analyzed structural complexity by means of the Sholl analysis. Neurons derived from hippocampal primary culture become appropriately polarized, pass though defined stages of maturation (Dotti et al., 1988) and develop synaptic connections (Soriano et al., 2008; Verderio et al., 1999). Hippocampal primary culture is a commonly used model system for addressing a wide range of questions in molecular and cellular neurobiology (Grabrucker et al., 2009). At DIV7 in culture, excitatory synapses with a clearly defined postsynaptic density (PSD) and synaptic vesicles at the presynaptic membrane were found on dendrites/spines (Grabrucker et al., 2009). Moreover, at DIV7 axonal and dendritic outgrowth had already established and neurons start to mature (Dotti et al., 1988). Briefly, at DIV5 neurons were transfected with a plasmid encoding *EGFP* under the *Thy1* promoter, and at DIV7 we added nicotine (10 μM) to the medium for 48 hours, and studied the possible changes in structural complexity at DIV9, in nicotine-treated and untreated WT and transgenic neurons (see Methods). Repeated measures ANOVA yielded a significant genotype x treatment interaction [$F_{1,112} = 17.277$, $p < 0.001$] when analyzing the number of intersections arising from concentric circles with increasing radii (10 μm interspaced) extending from the soma. The analysis of the individual curves demonstrated that neurons derived from transgenic cultures were significantly less complex compared to neurons from control cultures ($p < 0.001$). Furthermore, nicotine treatment for 48 hours had opposed effects in WT and Tg*CHRNA5/A3/B4* cultures. The reduced structural complexity of Tg*CHRNA5/A3/B4* neurons was compensated by nicotine treatment ($p < 0.001$), whereas the same treatment significantly impaired structural complexity in WT neurons ($p < 0.001$) (Fig. 15 A and B). These results suggested that overexpression of the cluster *CHRNA5/A3/B4* alters the structural morphology of hippocampal neurons, an effect that is restored by nicotine treatment.

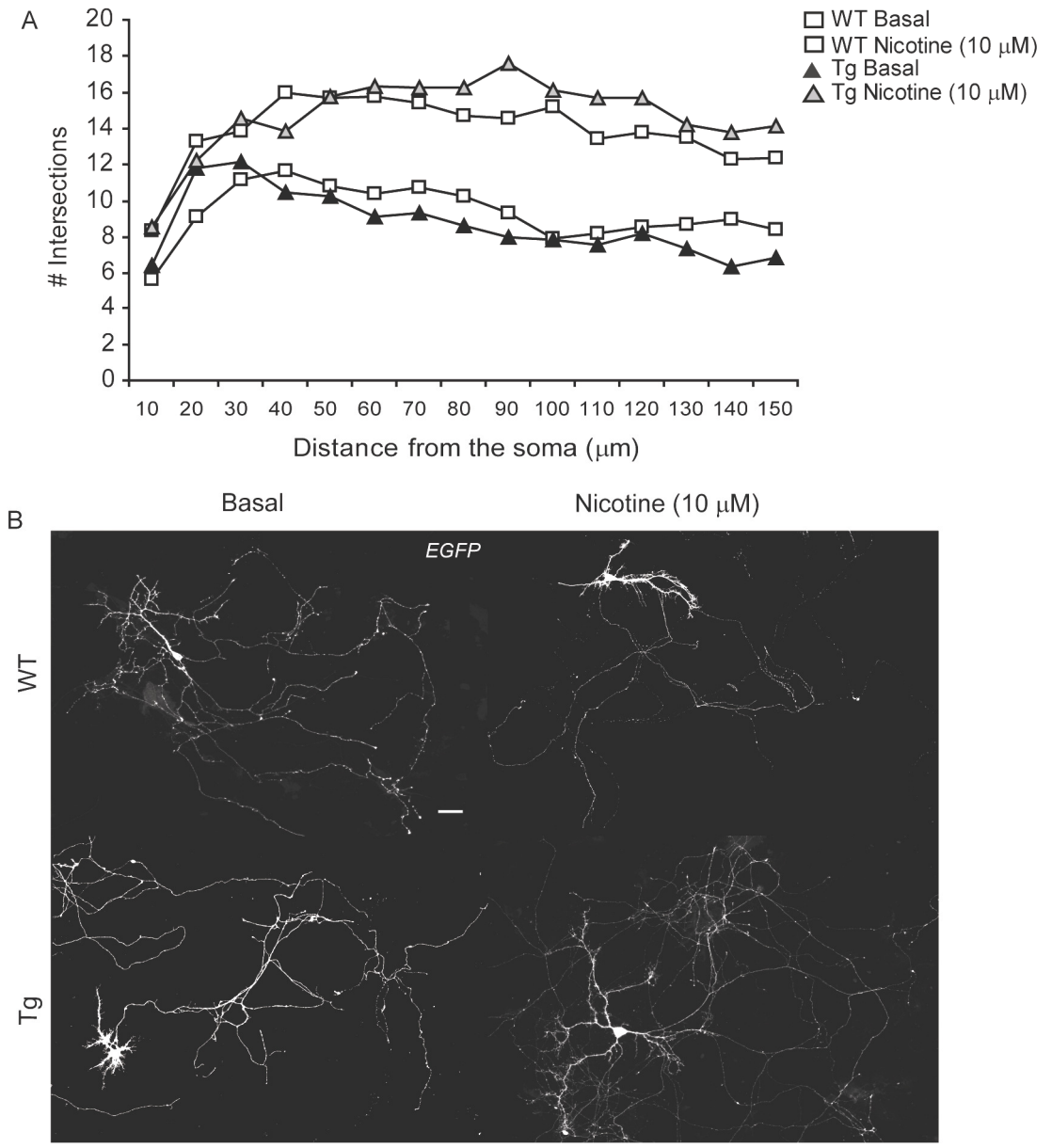


Figure 15. Tg*CHRNA5/A3/B4* pyramidal neurons show a significant reduction in structural complexity that is compensated with nicotine treatment. (A) Number of intersections arising from concentric circles with increasing radii (10 μm interspaced) extending from the soma (Sholl analysis) in WT cultures treated with medium (open squares) or medium with nicotine (10 μM)(gray squares) and Tg*CHRNA5/A3/B4* cultures treated with medium (filled triangle) or medium with nicotine (10 μM)(gray triangle) for 48 hours. **(B)** Photomicrograph illustrating positive neurons expressing *EGPF* from each group. Scale bar = 25 μm. N = 28 – 31 neurons per group, derived from at least 4 independent experiments. Data are expressed as mean.

To be sure that the differences observed in neuronal complexity were not due altered viability of the cell culture because of the cluster overexpression or toxic effects

of nicotine treatment, an MTT assay was performed. At DIV7 the medium was changed by fresh medium or medium with nicotine (10 μM) for 48 hours, until DIV9, when the MTT assay was carried out. Two-way ANOVA did not show significant differences between genotype, treatment and genotype x treatment interaction in relative absorbance at 550 nm (Table 3). This control experiment confirmed that overexpression of the cluster or nicotine treatment did not affect neuronal survival.

Genotype	Treatment	Relative Abs. (550 nm)
WT	Basal	1.00 ± 0.000
WT	Nicotine (10 μM)	0.84 ± 0.123
Tg*CHRNA5/A3/B4*	Basal	1.39 ± 0.297
Tg*CHRNA5/A3/B4*	Nicotine (10 μM)	1.24 ± 0.251

Table 3. Overexpression of the cluster *CHRNA5/A3/B4* and nicotine (10 μM) do not affect cell culture viability. Relative absorbance at 550 nm in a MTT assay in WT and Tg*CHRNA5/A3/B4* cultures treated with either basal medium or nicotine (10 μM). N = 6 independent experiments. Data are expressed as mean ± S.E.M. relative to the WT basal levels for each experiment.

4.3.3. Dendritic spine density in pyramidal neurons

Dendritic spines represent the postynaptic comparment for most glutamatergic synapses and may provide an anatomical substrate for memory storage and synaptic transmission. Dendritic spines are remarkably dynamic and can be divided into general categories such as stubby (type-I), mushroom-like (type-II), and thin (type-III), also filopodia-like structures, that are thought to reflect differences in functional properties (Bourne and Harris, 2008; Hotulainen and Hoogenraad, 2010). The spine density for each subtype was analyzed within a region of 30 μm proximal to the first branch point of primary dendrites in WT and Tg*CHRNA5/A3/B4* hippocampal cultures treated with medium or medium with nicotine (10 μM) for 48 hours. Two-way ANOVA revealed a significant effect of genotype [$F_{1,101} = 4.822$, $p < 0.05$] and treatment [$F_{1,101} = 8.109$, $p < 0.01$] in type-I (stubby) dendritic spines density (Fig. 16A and B). Neurons derived from transgenic cultures presented lower density of type-I spines compared to WT neurons and nicotine treatment significantly reduced the density of type-I spines in both genotypes. No significant differences were observed among the four groups in density of type-II spines, but two-way ANOVA revealed a significant increase in the density of

type-III spines [$F_{1,101} = 4.444$, $p < 0.05$] and filopodia [$F_{1,101} = 3.850$, $p < 0.05$] upon 48 hours nicotine exposure in both genotypes. Also, a significant genotype effect on filopodia density [$F_{1,101} = 14.275$, $p < 0.001$], was detected, Tg*CHRNA5/A3/B4* neurons presenting higher density of filopodia-like spines (Fig. 16A and B). Overexpression of the cluster *CHRNA5/A3/B4* and nicotine treatment produced similar effects on density of dendritic spines, increasing the number of thinner spines (filopodia and type-III spines) and reducing the density of type-I spines.

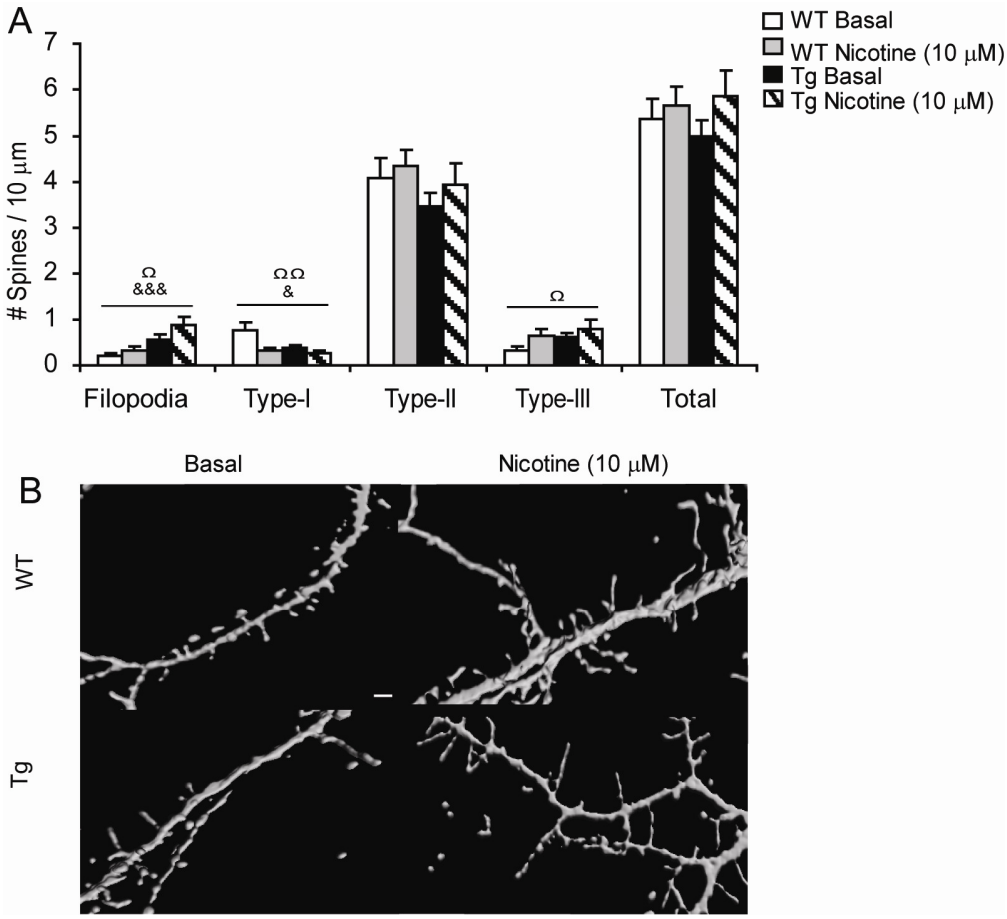


Figure 16. Effects of *CHRNA5/A3/B4* cluster overexpression and nicotine on the density of dendritic spines in hippocampal primary cultures. **(A)** Number of dendritic spines per 10 μm of dendritic length in WT neurons treated with medium (open) or medium with nicotine (10 μM)(gray) and Tg*CHRNA5/A3/B4* neurons treated with basal medium (filled) or medium with nicotine (10 μM)(striped) for 48h. **(B)** Photomicrograph illustrating dendritic spines from each group. Scale bar = 2 μm. N = 25 primary dendrites per group, derived neurons coming from at least 4 independent experiments. Data are expressed as mean ± S.E.M. Two-way ANOVA genotype effect & $p < 0.05$; &&& $p < 0.001$; or treatment effect Ω $p < 0.05$; Ω Ω $p < 0.01$.

4.3.4. Electrophysiological properties of the hippocampus: Paired-pulse facilitation and long-term potentiation (LTP)

The activation of nAChRs by nicotine is able to modulate synaptic plasticity in the hippocampus (Kenney and Gould, 2008a). Indeed, nicotine is able to shift short-term potentiation (STP) to long-term potentiation (LTP) in brain circuits involved in reinforcement and reward processing (Ji et al., 2001; Mansvelder and McGehee, 2000). The following experiments were undertaken to test whether overexpression of the cluster *CHRNA5/A3/B4* altered functional synaptic connection.

Extracellular field excitatory postsynaptic potentials (fEPSPs) evoked by Schaffer collateral stimulation were recorded from stratum radiatum of the dorsal CA1 hippocampal region in slices obtained from WT and Tg*CHRNA5/A3/B4* mice. In a first set of experiments we examined paired pulse facilitation ratio (PPR), a short-term presynaptic phenomenon that at CA3-CA1 synapses is directly related to the probability of neurotransmitter release (Zucker, 1989). Paired-pulse facilitation was induced by a double-pulse (30 ms apart) stimulation protocol and it was evaluated by calculating the paired-pulse facilitation ratio (PPR) between the second ($fEPSP_2$) and the first ($fEPSP_1$) responses. Hippocampal slices of WT and Tg*CHRNA5/A3/B4* mice were maintained in artificial cerebrospinal fluid (ACSF) medium alone or containing nicotine (100 μM) for 20 minutes and after washing the PPR was evaluated. In all the cases, the fEPSPs evoked by a second stimulus was facilitated. Interestingly, two-way ANOVA resulted in a significant genotype x treatment interaction [$F_{1,76} = 4.834$, $p < 0.05$] in PPR. Bonferroni *post hoc* comparisons between individual groups indicated no differences between WT and Tg*CHRNA5/A3/B4* slices. However, WT nicotine-treated hippocampal slices presented a significantly reduced PPR compared to nicotine-treated transgenic slices ($p < 0.001$) (Fig. 17), revealing opposite nicotine effects depending on the genotype. The results suggest that overexpression of the cluster *CHRNA5/A3/B4* increases the nicotine-induced probability of glutamate release from the Shaffer collaterals, while nicotine treatment decreases this probability in WT mice.

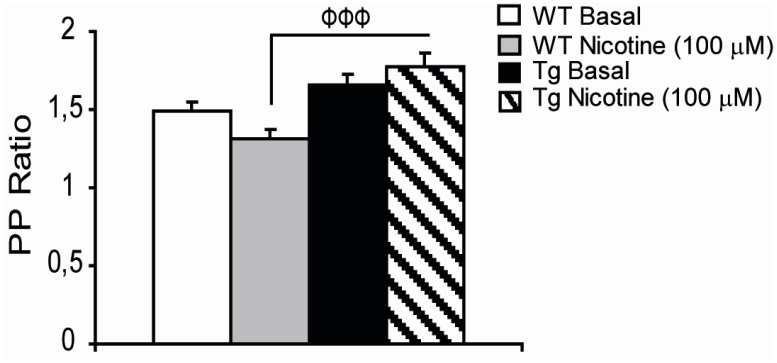


Figure 17. Genotype-specific nicotine effects on paired pulse facilitation ratio. Paired-pulse facilitation ratio (PPR) was induced by a double-pulse (30 ms apart) stimulation protocol and evaluated by calculating the paired-pulse ratio (PPR) between the slope of the second ($fEPSP_2$) and the first ($fEPSP_1$) responses from the Schaffer collateral pathway. PPR in WT hippocampal slices treated with basal ASCF medium (open) or medium with nicotine (100 μM)(gray) and Tg*CHRNA5/A3/B4* hippocampal slices treated with basal ASCF medium (filled) or medium with nicotine (100 μM)(striped) for 20 min. N = 15-25 slices per group. Data are expressed as mean ± S.E.M. Two-way ANOVA genotype x treatment interaction ($p < 0.05$). Bonferroni *post hoc* comparisons between genotype (ΦΦΦ $p < 0.001$).

The next series of experiments tested LTP in the hippocampus of Tg*CHRNA5/A3/B4* mice and how it was modified by nicotine treatment. LTP was induced by high-frequency stimulation (HFS) (five trains of 100 pulses at 100 Hz delivered at 10-s interval) and registered for 60 minutes (pulse at 0.03 Hz). LTP was induced in all experimental groups (Fig. 18A and B). In Tg*CHRNA5/A3/B4* slices, the degree of potentiation was significantly reduced as compared to the WT. Repeated measures ANOVA yielded a significant genotype effect [$F_{1,27} = 8.944$, $p < 0.01$] when measuring LTP immediately (5 min) and 45 minutes after HFS application. Nicotine increased LTP in WT but it did not affect Tg*CHRNA5/A3/B4* response to HFS. These results suggested that at CA3-CA1 synapses of mice overexpressing the cluster *CHRNA5/A3/B4* long-term synaptic plasticity processes are affected.

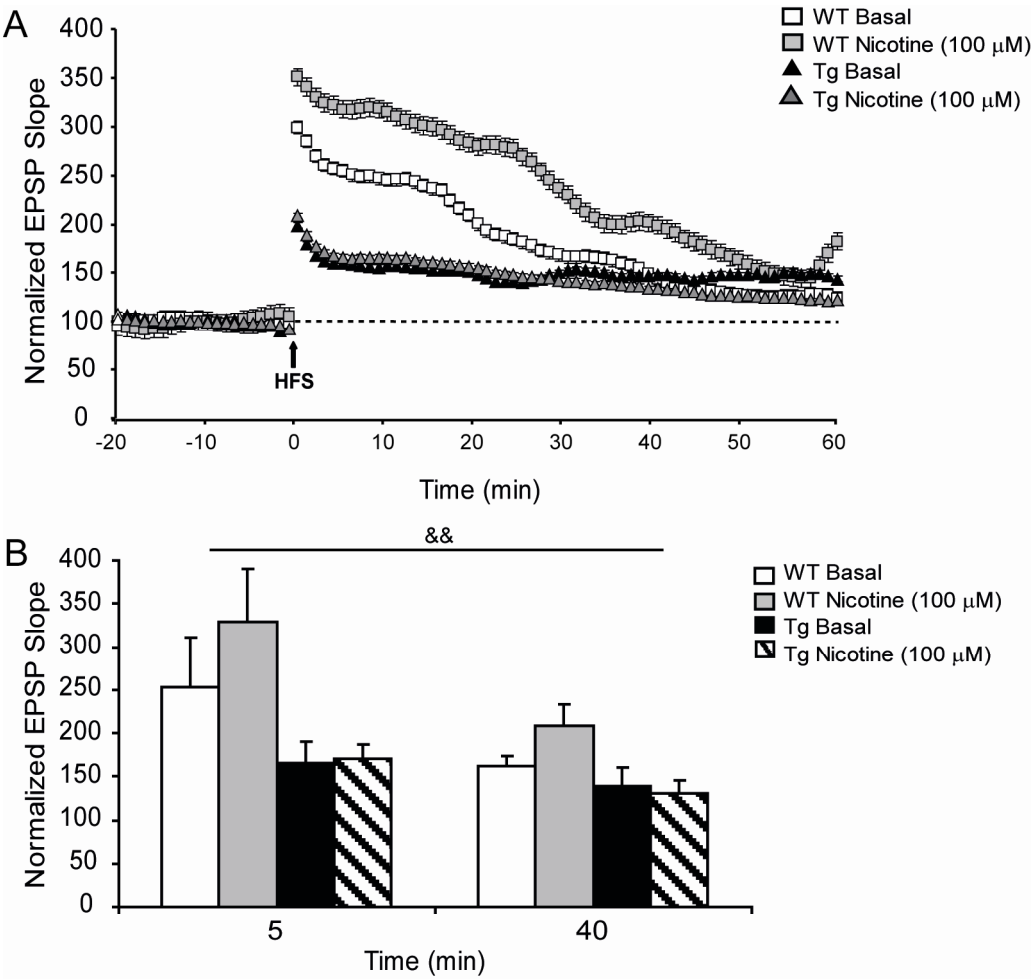


Figure 18. Overexpression of the cluster *CHRNA5/A3/B4* reduces HFS-induced LTP. (A) LTP induced by five HFS trains (1 s at 100Hz, 10 s apart) delivered to the Schaffer collateral. The slopes of the fEPSPs were normalized to those obtained before HFS. Each point is the average of five minutes obtained from WT hippocampal slices treated with basal ASCF medium (open square) or medium with nicotine (100 μM)(gray square) and Tg*CHRNA5/A3/B4* hippocampal slices treated with basal ASCF medium (filled triangle) or medium with nicotine (100 μM)(gray triangle) for 20 min. **(B)** Amount of potentiation measured 5 and 45 minutes after LTP induction in WT hippocampal slices treated with basal ASCF medium (open) or medium with nicotine (100 μM)(gray) and Tg*CHRNA5/A3/B4* hippocampal slices treated with basal ASCF medium (filled) or medium with nicotine (100 μM)(striped) for 20 min. N = 7-10 slices per group. Data are expressed as mean ± S.E.M. ANOVA repeated measures genotype effect (&& $p < 0.01$).

4.4. CHARACTERIZATION OF THE NICOTINE ADDICTIVE PROFILE IN Tg*CHRNA5/A3/B4* MICE

The last part of the Thesis was addressed to specific aspects of nicotine addiction such as self-administration, tolerance and withdrawal. Also, nicotine-induced activation of regions involved in the rewarding and aversive properties of nicotine, such as the VTA and habenula (MHb and LHb), was determined using c-Fos protein expression analysis.

4.4.1. The two-bottle choice paradigm

Our previous results demonstrated increased reinforcing effects of nicotine in Tg*CHRNA5/A3/B4* mice, in a simple fixed-ratio schedule of intravenous nicotine self-administration (Gallego et al., 2011). Here we used the two-bottle choice paradigm to investigate how overexpression of the cluster *CHRNA5/A3/B4* modified the pattern of oral nicotine self-administration at increasing doses of the drug.

Experiment 1

First, oral self-administration was characterized when low doses of nicotine (1 and 5 μg/ml) were available in drinking water. ANOVA repeated measures on consumption data (mg/kg/day) indicated a significant dose effect when the dose increased from 1 to 5 μg/ml of nicotine [$F_{1,18} = 142.861$, $p < 0.001$] with no significant differences between genotypes (Fig. 19A). However, a trend to higher preference for nicotine in Tg*CHRNA5/A3/B4* mice was detected, although it did not reach statistical significance (Fig. 19B). Control experiments demonstrated that total volume consumed remained stable along days and no significant differences were observed between genotypes (Fig. 19C).

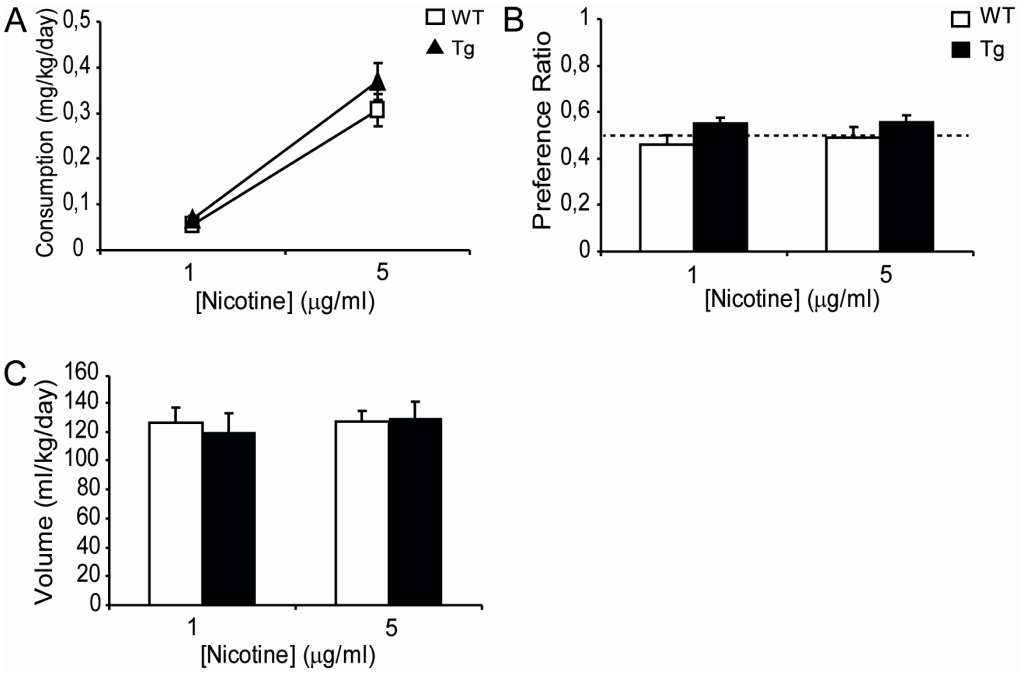


Figure 19. Nicotine preference and consumption in the two-bottle choice paradigm at very low doses of the drug. (A) Nicotine consumption (mg/kg/day) and **(B)** preference (volume of nicotine drinking relative to total fluid intake; ml nicotine / ml total) in a two-bottle choice paradigm in WT (open) and Tg*CHRNA5/A3/B4* (filled) mice. **(C)** Total fluid intake (ml/kg/day) control. Each dose represented the average of four consecutive days. N = 8-11 mice per group. Data are expressed as mean ± S.E.M.

Experiment 2

In a second set of experiments nicotine consumption was analyzed over 6 doses ranged from 10, 20, 35, 50, 100 to 200 μg/ml. Repeated measures ANOVA of consumption data (mg/kg/day) showed a significant effect of dose [$F_{5,27} = 42.027$, $p < 0.001$] and a significant dose x genotype interaction [$F_{5,27} = 3.838$, $p < 0.05$]. Student's T test statistics for each individual dose revealed significant differences between genotypes only at the highest dose of nicotine tested (200 μg/ml) [$t_{1,27} = 2.410$, $p < 0.05$] (Fig. 20A). Regarding the preference data, there was a significant dose effect [$F_{5,27} = 32.301$, $p < 0.001$], but no genotype effect (Fig. 20B). Total fluid intake showed no differences in any condition (Fig. 20C). Furthermore, control experiments for sweetened or bitter taste demonstrated that the overexpression of the cluster *CHRNA5/A3/B4* did not influence taste sensitivity, since WT and Tg*CHRNA5/A3/B4* mice had similar preference for 0.2% saccharin or aversion for 0.5 mM quinine (Fig. 20D).

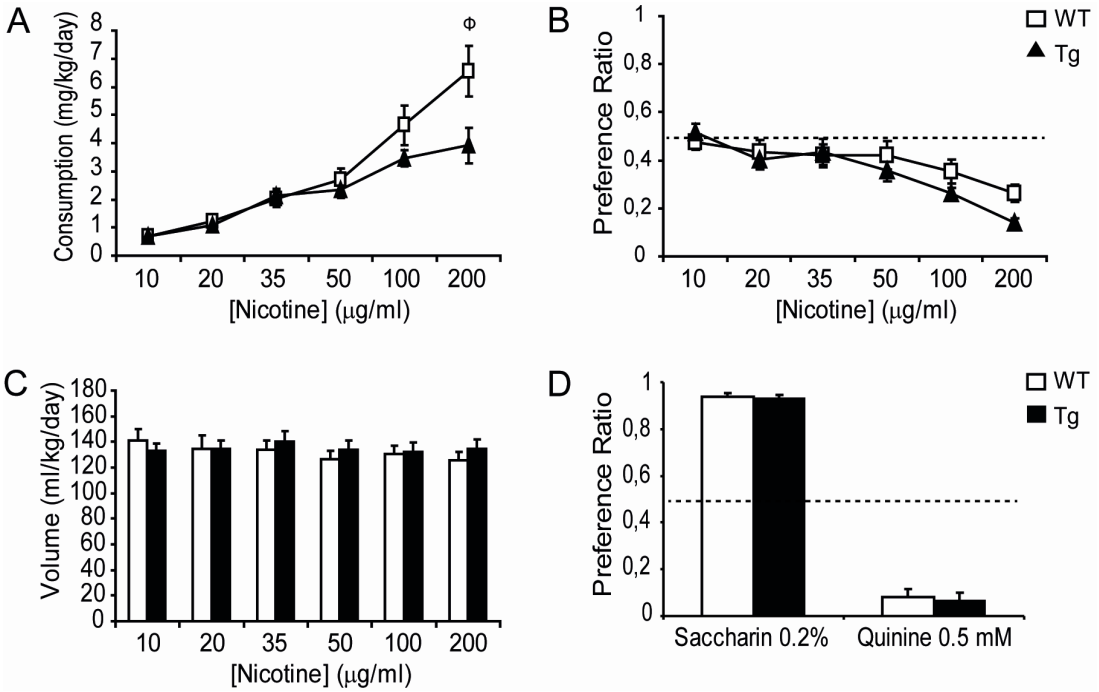


Figure 20. Tg*CHRNA5/A3/B4* mice show reduced consumption of high doses of nicotine (200 μg/ml). **(A)** Nicotine consumption (mg/kg/day) and **(B)** preference (volume of nicotine drinking relative to total fluid intake; ml nicotine / ml total) in a two-bottle choice paradigm between water and water containing the indicated nicotine concentration, in WT (open) and Tg*CHRNA5/A3/B4* (filled) mice. **(C)** Total fluid intake (ml/kg/day) control. **(D)** Preference ratio for saccharin (0.2%) and quinine (0.5 mM). Each dose represented the average of four consecutive days. N = 13-16 mice per group. Data are expressed as mean ± S.E.M. Student's T test Φ $p < 0.05$.

Experiment 3

In the last set of experiment we studied whether a single exposure to a high dose of nicotine was able to modify the subsequent pattern of nicotine self-administration. To this aim, mice were habituated to the cages for 4 days receiving only water, and then one of the bottles was replaced by water containing nicotine (10^3 μg/ml) for 12 hours. They returned to tap water for 3 days before exposure to increasing doses of nicotine. Student's T test showed no significant differences between genotypes regarding the amount of nicotine consumed (mg/kg/day) (Fig. 21A) or the preference ratio (Fig. 21B) when exposed to a high dose of nicotine. When returned to tap water, Tg*CHRNA5/A3/B4* animals avoided drinking liquid from the bottle that previously contained nicotine (not shown). ANOVA repeated measures on consumption (mg/kg/day) yielded a significant effect of dose [$F_{5,26} = 36.357$, $p < 0.001$] and also a significant genotype x dose interaction [$F_{5,26} = 8.737$, $p < 0.001$] in mice pre-exposed to

a high dose of nicotine (Fig. 21C). Student's T test for each dose demonstrated a significant genotype effect at 50 µg/ml [$t_{1,26}$ = 2. 690, $p < 0.05$] and 200 µg/ml [$t_{1,26}$ = 4.413, $p < 0.001$]. As for nicotine preference, there was a significant effect of dose [$F_{5,26}$ = 23.319, $p < 0.001$] and a significant effect of genotype [$F_{1,26}$ = 4.199, $p < 0.05$] since Tg*CHRNA5/A3/B4* mice show a reduced preference for nicotine compared to their WT littermates. However, the dose x genotype interaction was not significant (Fig. 21D). Again, total fluid intake remained stable along increasing concentrations of nicotine and there were no significant differences between genotypes (Fig. 21E). Furthermore, control experiments for sweet or bitter taste demonstrated that neither overexpression of the cluster *CHRNA5/A3/B4* nor the exposure to a single high dose of nicotine influenced taste. No significant differences between genotypes regarding preference for 0.2% saccharin or aversion to 0.5 mM quinine existed (Fig. 21F). Importantly, the present results indicated that a single exposure to a high dose of nicotine was sufficient to increase aversion to high doses of nicotine in mice overexpressing the cluster *CHRNA5/A3/B4* but not in WT animals. In Tg*CHRNA5/A3/B4* mice, repeated measures ANOVA revealed a significant dose x group interaction [$F_{1,25}$ = 3.420, $p < 0.05$] in nicotine consumption (mg/kg/day). Student's T test analysis for each individual dose demonstrated significant differences between the non pre-exposed and the pre-exposed groups of Tg*CHRNA5/A3/B4* mice at the doses of 10 µg/ml [$t_{1,25}$ = -2.783, $p < 0.01$], 20 µg/ml [$t_{1,25}$ = -2.998, $p < 0.01$], 35 µg/ml [$t_{1,25}$ = -2.521, $p < 0.05$] and 200 µg/ml [$t_{1,25}$ = -3.157, $p < 0.01$]. For the preference data, no significant differences were observed between the two groups of Tg*CHRNA5/A3/B4* mice.

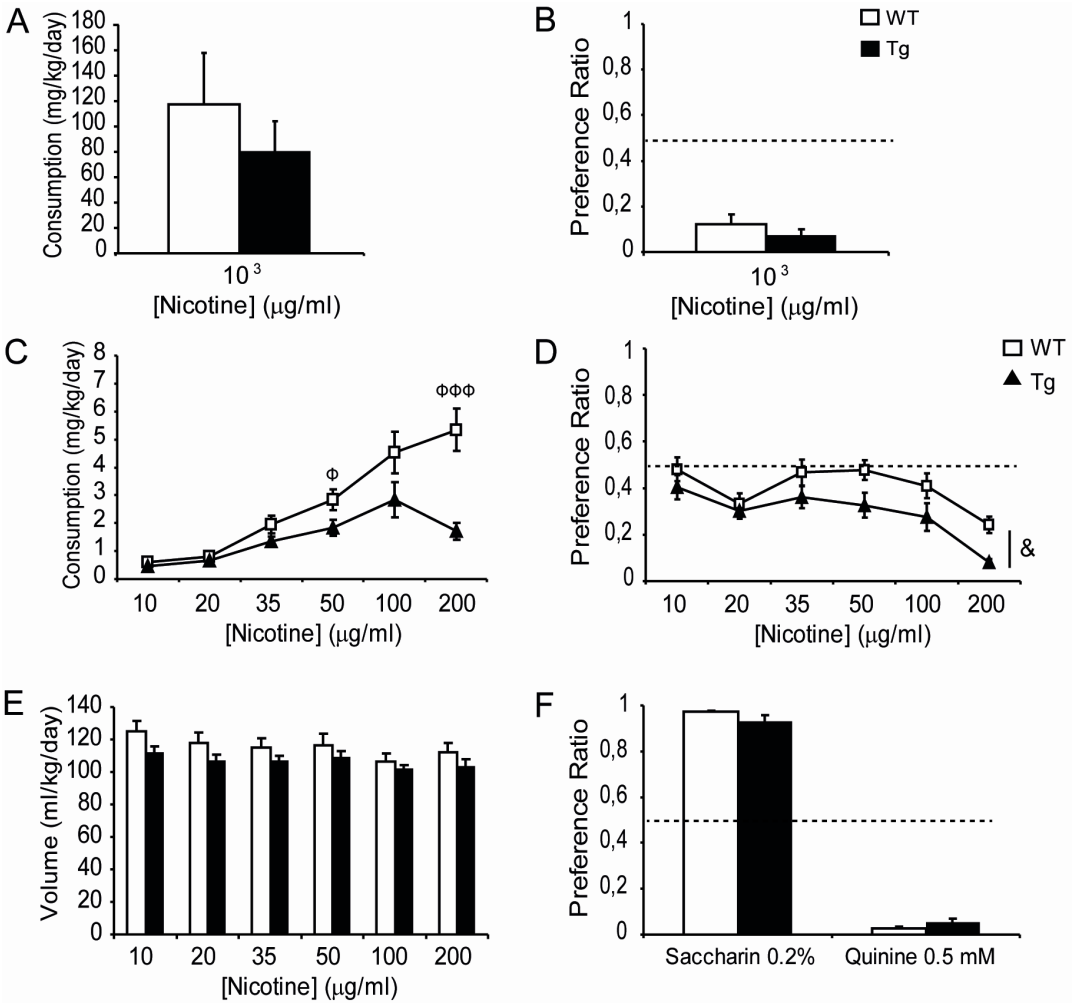


Figure 21. Pre-exposure to a high dose of nicotine (10^3 µg/ml) increases the aversion to high doses of nicotine in Tg*CHRNA5/A5/B4* mice. (A) Nicotine consumption (mg/kg/day) and **(B)** preference ratio (volume of nicotine drinking relative to total fluid intake; ml nicotine / ml total) during the 12 hours exposure to a high dose of nicotine. Oral nicotine self-administration when administered in a wide range of doses expressed as consumption (mg/kg/day) **(C)** or preference ratio **(D)**. Total fluid intake (ml/kg/day) control **(E).** Preference ratio for saccharin (0.2%) and quinine (0.5 mM) **(F)**. Each dose represented the mean of four consecutive days in WT (open) and Tg*CHRNA5/A3/B4* (filled) mice. N = 14 mice per group. Data are expressed as mean ± S.E.M. In consumption data, ANOVA repeated measures genotype x dose interaction ($p < 0.05$); Student's T test Φ $p < 0.05$; ΦΦΦ $p < 0.001$. ANOVA repeated measures on preference ratio: genotype effect & $p < 0.05$.

4.4.2. Nicotine-induced withdrawal syndrome

Cessation of chronic nicotine treatment (spontaneous) and acute challenge with various nicotinic antagonists (precipitated) are two distinct ways to induce withdrawal.

Mecamylamine injected to animals implanted with nicotine-filled minipumps represents a highly reproducible method to precipitate withdrawal syndrome (Damaj et al., 2003). Mice were chronically treated with either saline or nicotine (10 mg/kg/d) for 7 days and withdrawal syndrome was precipitated by acute injection of mecamylamine (1 mg/kg). Immediately after injection, mice were observed for manifestation of somatic signs of withdrawal during 20 min. Two-way ANOVA revealed that mice chronically treated with nicotine exhibited significantly more somatic signs of withdrawal than saline-treated mice [treatment effect, $F_{1,23} = 4.291$, $p < 0.05$] (Fig. 22A). However, no significant differences between genotypes were observed (genotype x treatment interaction, n.s.), suggesting that nicotine-treated Tg*CHRNA5/A3/B4* mice displayed withdrawal scores similar to nicotine-treated WT mice after mecamylamine injection.

Because mecamylamine is a general antagonist for the nAChRs but has a higher affinity for $\alpha 3\beta 4^*$ containing receptors (Papke et al., 2001), it might be possible that Tg*CHRNA5/A3/B4* mice showed different sensitivity to mecamylamine compared to their WT littermates. In order to investigate this possibility, a second study was carried out in mice chronically treated with either saline or nicotine (10 mg/kg/d) for 7 days, in which the minipump was explanted on day 7, and spontaneous withdrawal was evaluated 24 hours after minipump removal. As shown in Fig. 22B, there was a significant genotype x treatment interaction [$F_{1,54} = 3.116$, $p = 0.084$] in general somatic signs of withdrawal. Bonferroni *post hoc* comparisons between individual groups demonstrated that nicotine-treated Tg*CHRNA5/A3/B4* mice exhibited significantly increased somatic signs of withdrawal compared to nicotine-treated WT mice ($p < 0.05$) and obviously to saline-treated Tg*CHRNA5/A3/B4* animals ($p < 0.001$).

When affective signs of nicotine-induced withdrawal were evaluated, two-way ANOVA did not show significant differences between groups, neither in mecamylamine-precipitated withdrawal (Fig. 22C) nor in spontaneous withdrawal (Fig. 22D). The dose and duration of nicotine treatment were not sufficient to alter the percentage of time that mice spent in aversive compartments in non of the groups, which is considered to be an index of anxiety-like behaviour, and a measure of affective signs of withdrawal. Additionally, we evaluated whether withdrawal from chronic nicotine either precipitated by mecamylamine injection or spontaneous induced, increased the sensitivity to nociceptive stimuli. To this aim, after evaluating the affective component of withdrawal, hyperalgesia was examined using the hot-plate test.

Two-way ANOVA analysis revealed that neither precipitated nor spontaneous nicotine withdrawal modified the latency to paw lick or latency to jump in none of the groups (Fig. 22E,F).

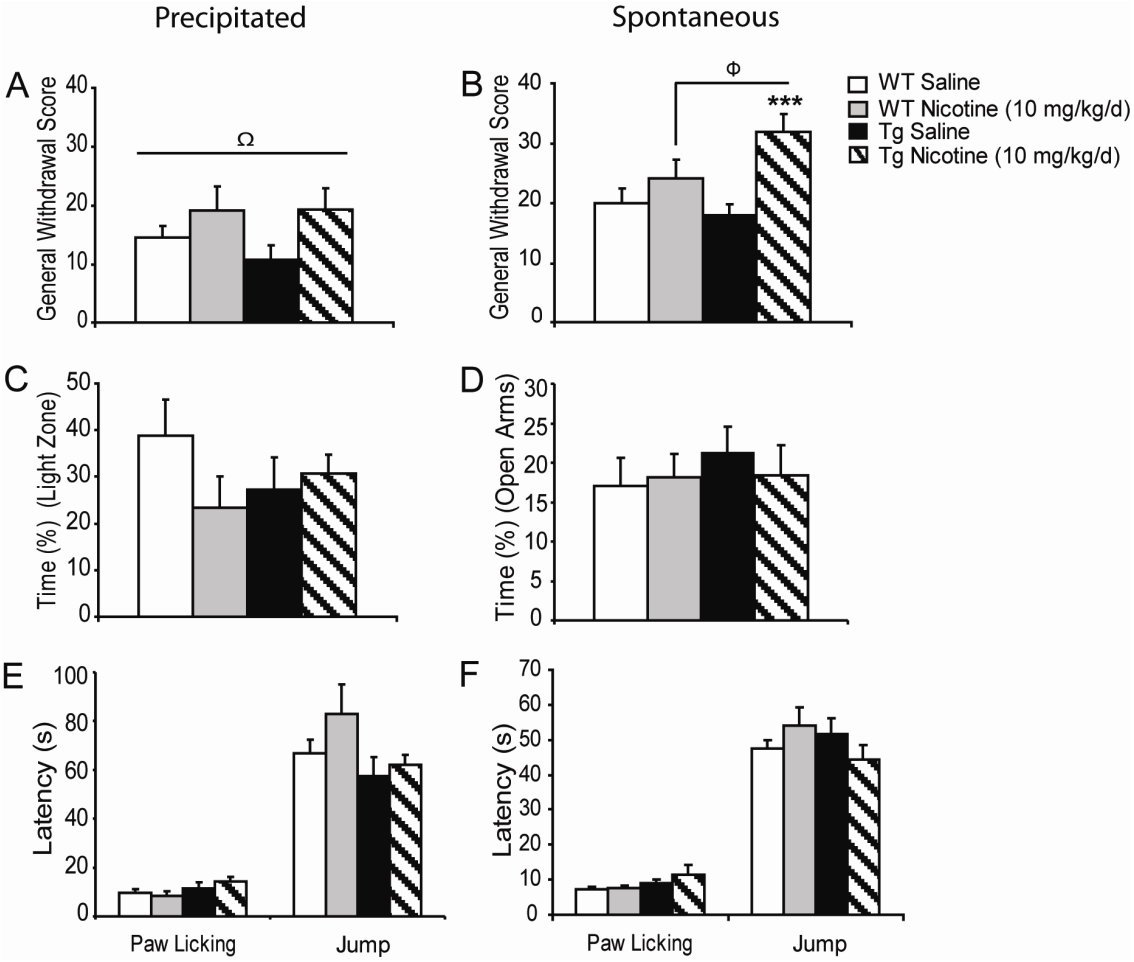


Figure 22. Tg*CHRNA5/A3/B4* mice show increased spontaneous but not mecamylamine precipitated nicotine-induced withdrawal signs. General withdrawal score of somatic signs in saline (open) or nicotine (gray) treated WT mice and saline (filled) or nicotine (striped) treated Tg*CHRNA5/A3/B4* when withdrawal was precipitated with mecamylamine (N = 5 - 6 mice per group) **(A)** or spontaneously-induced (N = 12-15 mice per group) **(B).** Affective signs of nicotine withdrawal represented as percentage of time spent in the aversive compartment of the light and dark box when withdrawal was precipitated with mecamylamine **(C)** or in the unprotected arms or the elevated plus maze when withdrawal was spontaneously-induced **(D).** Changes in nociception measured as latency to paw licking or jump in the hot plate test in mecamylamine-precipitated **(E)** or spontaneously-induced **(F)** withdrawal. Data are expressed as mean ± S.E.M. In precipitated withdrawal Two-way ANOVA treatment effect (Ω $p < 0.05$); in spontaneous withdrawal genotype x treatment interaction ($p < 0.05$) Bonferroni *post hoc* comparisons between treatment (** $p < 0.01$) and between genotype (Φ $p < 0.05$).

4.4.3. Nicotine-induced tolerance

We examined whether the chronic nicotine treatment selected was able to induce tolerance to the effects of nicotine, which is fundamental for the development of drug dependence. To this aim, after 7 days of chronic treatment with either saline or nicotine (10 mg/k/d) mice received an acute injection of a high dose of nicotine (6 mg/kg) and were evaluated for the manifestation of nicotine-induced seizures. Two-way ANOVA statistics confirmed that Tg*CHRNA5/A3/B4* mice were hypersensitive to the acute effects of nicotine, indeed, Tg*CHRNA5/A3/B4* animals showed a high seizure score compared to their WT littermates [genotype effect, $F_{1,16} = 17.918$, $p < 0.001$]. However, the same statistics demonstrated no differences between saline and nicotine treated groups, neither genotype x treatment interaction, although nicotine-treated animals tended to have less seizures compared to the saline-treated counterparts (Fig. 23A). It should be reminded (see above) that the dose of nicotine of 6 mg/kg elicited clonic/tonic seizures only in Tg*CHRNA5/A3/B4* animals (Fig. 23B). Additionally, also it was analyzed if the latency to elicit seizures was modified when animals were chronically treated with nicotine. Student's T test showed that Tg*CHRNA5/A3/B4* mice chronically treated with nicotine tended to elicit myoclonic seizures later in time compared to the saline-treated Tg*CHRNA5/A3/B4* animals, although it did not reached statistically significance [$t_{1,6} = -2.186$, $p = 0.071$] (Fig. 23C). The same occurred to WT animals, with a non-significant tendency in nicotine-treated control mice to exhibit seizures later in time compared to the saline-treated group [$t_{1,5} = -2.297$, $p = 0.093$] (Fig. 23D).

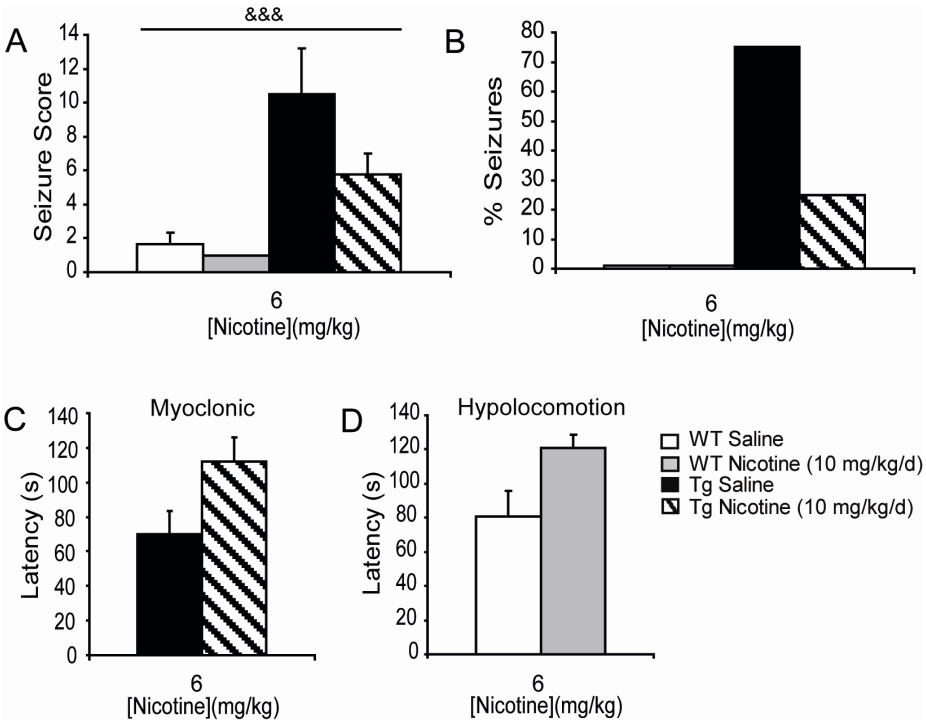


Figure 23. Effect of chronic infusion of 10 mg/kg/d nicotine for 7 days on nicotine-induced seizures. Seizure score **(A)** and percentage of mice that displayed clonic/tonic seizures **(B)** after acute injection of 6 mg/kg nicotine in WT mice chronically treated for 7 days with saline (open) or nicotine (10 mg/kg/d)(gray) and Tg*CHRNA5/A3/B4* mice that received saline (filled) or nicotine (10 mg/kg/d)(striped) for 7 days. **(C)** Latency to elicit myoclonic seizures in Tg*CHRNA5/A3/B4* mice and **(D)** latency to elicit locomotor alterations in WT mice. N = 3-5 mice per group. Data are expressed as mean ± S.E.M. Two-way ANOVA genotype effect (&&& $p<0.05$).

4.4.4. Nicotine-induced activation of the VTA, MHb and LHb: c-Fos expression analysis

From the structural point of view, the brain regions mainly involved in the rewarding and aversive effects of nicotine are the VTA and the habenula, respectively. By using c-Fos expression analysis we studied the activation of the VTA, MHb and LHb after an acute injection of 0.5 mg/kg of nicotine, a dose reported to induce place preference in mice and increase the firing rate of DAergic neurons of the VTA (Grenhoff et al., 1986). In the VTA, two-way ANOVA revealed a significant genotype x treatment interaction [$F_{1,15} = 23.5$, $p < 0.001$] in the number of double c-Fos/TH-positive nuclei. Bonferroni *post hoc* comparisons indicated that nicotine-injected groups showed increased expression of c-Fos in TH positive neurons compared to saline-treated groups both in WT ($p < 0.001$) and Tg*CHRNA5/A3/B4* animals ($p < 0.01$) (Fig.

24A,B). However, c-Fos expression was significantly reduced in nicotine-injected Tg*CHRNA5/A3/B4* mice as compared to nicotine-injected WT littermates ($p < 0.001$). These differences were not attributable to morphometrical alterations in the VTA of Tg*CHRNA5/A3/B4* animals since the number of TH positive neurons and total neurons in the VTA were similar between genotypes (not shown). Regarding the activation of the MHb, two-way ANOVA indicated that the same dose of nicotine of 0.5 mg/kg increased the number of c-Fos positive nuclei in both genotypes [treatment effect; $F_{1,17} = 5.643$, $p < 0.05$], but no significant interaction genotype x treatment was observed (Fig. 24C). In contrast, the LHb was not activated by nicotine administration; we did not found genotype nor treatment effects, neither genotype x treatment interaction (Fig. 24D).

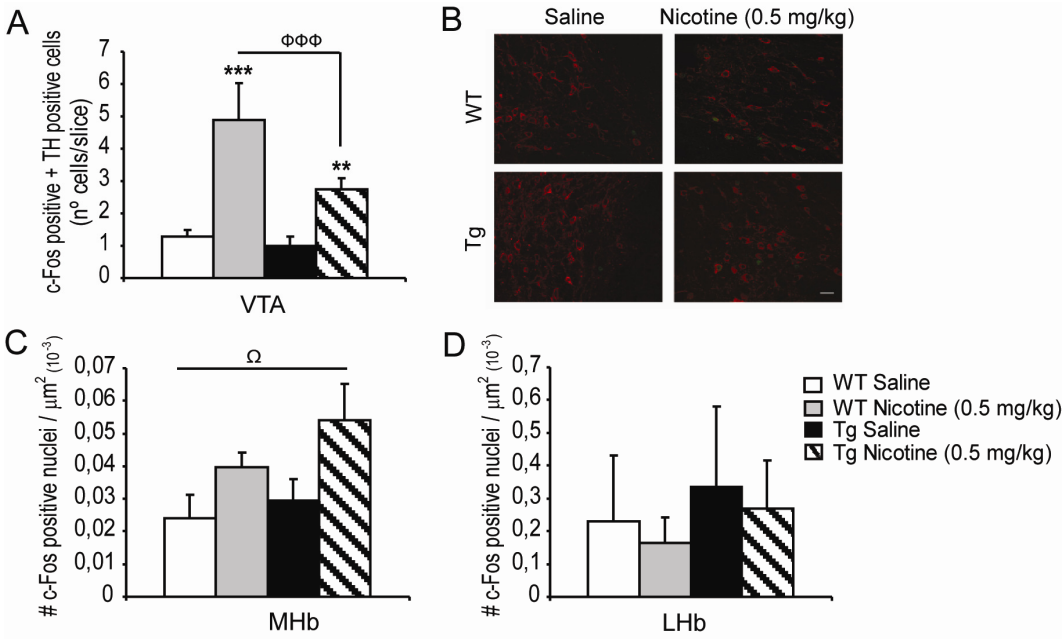


Figure 24. Tg*CHRNA5/A3/B4* mice show significantly reduced expression of c-Fos in DAergic neurons of the VTA after acute 0.5 mg/kg nicotine administration. (A) Quantification of double c-Fos / TH positive cells per slice in the VTA of WT mice that received either saline (open) or nicotine (0.5 mg/kg)(gray) and Tg*CHRNA5/A3/B4* mice treated with saline (filled) or nicotine (0.5 mg/kg)(striped). **(B)** Photomicrograph illustrating c-Fos immunoreactive DAergic cells in the VTA. Slices were fluorescently double-labelled with c-Fos (green) and Tyrosine hydroxilase (TH, red). Scale bar = 20µm. **(C)** In the MHb and **(D)** in the LHb c-Fos positive per area (μm^2) in WT mice that received either saline (open) or nicotine (0.5 mg/kg)(gray) and Tg*CHRNA5/A3/B4* mice treated with saline (filled) or nicotine (0.5 mg/kg)(striped) and sacrificed 120 minutes later for c-Fos expression analysis. N= 4 mice per group. Data are expressed as mean ± S.E.M. Two-way ANOVA treatment effect (Ω $p < 0.05$); Two-way ANOVA genotype x treatment interaction ($p < 0.05$). Bonferroni *post hoc* comparisons between treatment (** $p < 0.01$; *** $p < 0.001$) and between genotype (ΦΦΦ $p < 0.001$).

DISCUSSION

5. DISCUSSION

Human genetic studies have demonstrated that polymorphisms in the *CHRNA5/A3/B4* genomic cluster located on chromosome 15q24-25 may influence smoking-related behaviours. Some of these variants give rise to increased mRNA levels of specific subunits suggesting that transcriptional deregulation of the encoded nAChRs subunits may be involved in nicotine addiction (Falvella et al., 2010; Schlaepfer et al., 2008; Wang et al., 2009; Xu et al., 2006). Previous studies have explored the role of single receptor subunits by knocking out or overexpressing individual mouse nAChRs subunits. Here we used a BAC transgenic mouse model overexpressing the whole human genomic cluster *CHRNA5/A3/B4* (Tg*CHRNA5/A3/B4*) to explore the mechanism by which a deregulated expression of the α5, α3 and β4 subunits of the nAChRs could predispose to nicotine addiction. Our previous studies (Gallego et al., 2011; Gallego et al., 2012) showed that overexpression of the *CHRNA5/A3/B4* cluster increased nicotine binding sites in specific brain areas, leading to increased sensitivity to the pharmacological effects of nicotine. Several evidences indicate that the acute effects of nicotine may provide clues about the mechanism of tobacco addiction. Thus, understanding the mechanisms involved in this increased sensitivity is particularly relevant. We also explored whether overexpression of the cluster *CHRNA5/A3/B4* results in modifications in some cognitive domains associated to nicotine addiction, which may prone an individual to initiation and maintenance of smoking-related behaviours. To get insight into the possible link between the cognitive/addictive behaviours we studied how overexpression of the cluster *CHRNA5/A3/B4* altered morphological and functional characteristics of the hippocampus, as an important substrate for both nicotine addiction and cognition. Finally, our work addressed the addictive profile of mice overexpressing the cluster *CHRNA5/A3/B4* regarding nicotine self-administration and nicotine dependence.

5.1. CHARACTERIZATION OF THE PHARMACOLOGICAL EFFECTS OF NICOTINE IN Tg*CHRNA5/A3/B4* MICE

Our previous work showed that overexpression of the *CHRNA3/A5/B4* cluster affected specific pharmacological responses to nicotine. We wanted to proof if this

increased sensitivity was generalized to other nicotine-induced effects, such as changes in locomotion. In our experiments, acute nicotine administration significantly reduced locomotor activity in Tg*CHRNA5/A3/B4* mice compared to their WT littermates, at the dose of 1 mg/kg. This result confirm previous reports demonstrating that deletion of the α3, β4 or α5 subunits individually, or in combination, results in a phenotype resistant to acute nicotine-induced hypolocomotion (Jackson et al., 2010; Salas et al., 2004a; Salas et al., 2003). The increased α3β4* binding sites in the MHb of Tg*CHRNA5/A3/B4* animals compared to their WT littermates (Gallego et al., 2011) could be responsible for this effect, since the habenula-interpeduncular system is important for nicotine-induced hypolocomotion (Hentall, 1995). However, the effects of nicotine on locomotion are also mediated by DAergic neurons of the mesencephalon (VTA and substantia nigra pars compacta, SNc) (Salas et al., 2003), that express α5 and α3 subunits of the nAChRs (Wada et al., 1989). Since Tg*CHRNA5/A3/B4* mice also show increased nAChRs expression in these regions we cannot exclude that the VTA/SNc are involved in the increased sensitivity to nicotine. However, given that local infusion of nicotine or nicotinic agonists into the VTA results in increased locomotor activity (Leikola-Pelho and Jackson, 1992), and in our experiments we observe a more marked hypolocomotion effects elicited by nicotine, this mechanism seems a less feasible explanation.

Previous results in our laboratory demonstrated that overexpression of the *CHRNA5/A3/B4* cluster increases sensitivity to nicotine-induced seizures (Gallego et al., 2011). Sensitivity to nicotine-induced seizures is correlated with the number of hippocampal nAChRs binding sites (Marks et al., 1989; Miner and Collins, 1989), and deletion of the α3, β4 or α5 subunits individually, or in combination, results in a phenotype resistant to the acute nicotine seizures (Jackson et al., 2010; Kedmi et al., 2004; Salas et al., 2004a; Salas et al., 2003). We thus proposed that increased nAChRs was responsible for the increased sensitivity to nicotine-induced seizures in our transgenic mice. In our experiments, pre-treatment with a nAChRs antagonist (mecamylamine, 0.5 mg/kg) completely blocked nicotine seizures in WT mice as was also reported by Damaj (Damaj et al., 1999). However, it was able to only partially reduce nicotine-induced seizures in Tg*CHRNA5/A3/B4* mice. It could be argued that the concentration of mecamylamine used was not sufficient to block the nAChRs overexpressed in Tg*CHRNA5/A3/B4* animals, but a dose of 1 mg/kg of mecamylamine was neither capable to completely block nicotine seizures in Tg*CHRNA5/A3/B4* mice.

Even though mecamylamine markedly reduced the clonic/tonic component of seizures it did not block the appearance of myoclonic seizures or back arching in Tg*CHRNA5/A3/B4* mice. Thus, we proposed that nicotine might act through other non-cholinergic receptors to produce seizures in our Tg*CHRNA5/A3/B4* mice.

Several mechanisms have been proposed to explain seizures elicited by nicotine. Nicotine administration either directly or indirectly (though VGCC) produces a rise in intracellular free calcium that ultimately leads to the release of glutamate. The released glutamate can activate multiple postsynaptic receptors, such as NMDA receptors or AMPA/kainate receptors, although the NMDA subtype is known to be involved in seizures processes (Damaj et al., 1999). Interestingly, NMDA receptors antagonists such as MK-801, exhibit powerful anticonvulsant action restricted to generalized tonic-clonic seizures (Damaj et al., 1999; Mares et al., 2004). In accordance, MK-801 was effective in blocking seizures in WT but not in Tg*CHRNA5/A3/B4* mice in which it in fact aggravated the seizure phenotype, increasing circling and jumping behaviour. The interaction between MK-801 and nicotinic signalling has been previously described (Amador and Dani, 1991; Mastropaolo et al., 2004; Tizabi et al., 1998). Of interest to this discussion, nicotine or modulators of nicotinic neurotransmission attenuated MK-801-elicited explosive jumping (popping) behaviour in outbred mouse strains (Deutsch et al., 1995). In our Tg*CHRNA5/A3/B4* mice, nicotine accentuated MK-801-induced popping behaviour. Instead, a competitive AMPA/kainate receptors antagonist (CNQX) was also not able to block hypersensitivity to nicotine-induced seizures in Tg*CHRNA5/A3/B4* mice but in this case, it also did not reverse nicotine (24 mg/kg)-induced seizures in WT mice. Thus, our results suggest that nicotine induces seizures through activating postsynaptic NMDA but not AMPA/kainate receptors in WT mice but this does not seem to be supported in our Tg*CHRNA5/A3/B4* mouse, thus suggesting that the overexpression $\alpha 3$, $\beta 4$ or $\alpha 5$ subunits of the nAChRs may modify the influence of nicotine on NMDA receptor-mediated neurotransmission.

A relevant question that may help to explain the observed genotype-dependent seizure phenotypes is if the brain activation pattern is different upon overexpression of the cluster *CHRNA5/A3/B4*. We used c-Fos protein expression analysis to obtain a clear picture of the brain regions activated upon nicotine-induced seizures. Substantial evidence suggests that limbic structures such as the piriform cortex, entorhinal cortex and the hippocampus are major structures involved in seizures induced by various pro-

convulsive agents (i.e. PTZ, nicotine or NMDA) (Bastlund et al., 2005; Morgan and Linnoila, 1991). Moreover, electrophysiological studies have indicated that nicotine-induced seizures originate in the hippocampus (Floris et al., 1964), which is activated before the amygdala and cortex (Stitzel et al., 2000). The hippocampus receives rich cholinergic innervations, mainly from the medial septum-diagonal band complex (Dani and Bertrand, 2007), and nAChRs are highly expressed throughout the hippocampus (Albuquerque et al., 1997; Wada et al., 1989). The majority of these nAChRs subunits are expressed on hippocampal interneurons, although they are also found on pyramidal neurons (Alkondon and Albuquerque, 2001; Alkondon et al., 1996; Sudweeks and Yakel, 2000). Hippocampal nAChRs can be located presynaptically where they facilitate the release of several neurotransmitters, including glutamate, GABA and norepinephrine (Alkondon and Albuquerque, 2001; Wonnacott, 1997; Wonnacott et al., 1990) and on the soma of pyramidal cells and GABAergic neurons (Marks et al., 1996; Wada et al., 1990).

A dose of nicotine of 6 mg/kg (convulsive only in Tg*CHRNA5/A3/B4* mice) increased c-Fos positive nuclei in limbic regions, such as the CA1 SO and SR of the hippocampus and the piriform cortex exclusively in Tg*CHRNA5/A3/B4* mice. In the MHb (only the lateral part), VTA, and LC, 6 mg/kg of nicotine increased c-Fos expression in both Tg*CHRNA5/A3/B4* and WT mice. Since the nicotine dose used (6 mg/kg) was not convulsive in WT mice, these data suggest that overexpression of α3, β4 and α5 subunits of the nAChRs increases the sensitivity to nicotine-induced seizures through activation of the hippocampus and piriform cortex, but not the MHb, contradicting previous proposal (Jackson et al., 2010; Kedmi et al., 2004; Salas et al., 2004a; Salas et al., 2003).

Interestingly, the brain activation pattern of WT receiving a convulsive dose of nicotine (24 mg/kg) was slightly different to that observed in Tg*CHRNA5/A3/B4* mice upon convulsions. In WT, nicotine (24 mg/kg)-induced seizures increased c-Fos positive nuclei in the GCL of the hippocampus, MHb (both lateral and medial parts), also the piriform cortex and entorhinal cortex, compared with a non-convulsive dose of nicotine (6 mg/kg). Stimulation of the medial part of the MHb induces long-lasting increases in glutamate transmission in the IPN (Kawaja et al., 1990), involved in seizure activity in rodents and humans (Chiba et al., 1995). The activation of the medial part of the MHb upon nicotine convulsion only occurred in WT but not in Tg*CHRNA5/A3/B4*

mice, thus further supporting the idea that the habenula-interpeduncular nucleus is not involved in hypersensitivity to nicotine-induced seizures observed in Tg*CHRNA5/A3/B4* mice.

Taken together, our results indicate that the hippocampus and piriform cortex play major role in hypersensitivity to nicotine-induced seizures in Tg*CHRNA5/A3/B4* mice. However, it should be borne in mind that previous results in our laboratory (Gallego et al., 2011) indicated that Tg*CHRNA5/A3/B4* mice also show increased α3β4* nAChRs in other regions involved in limbic seizures, such as the olfactory bulb (OB), striatum, superior colliculus, dorsal tegmental nucleus and piriform cortex (Miller et al., 1991; Nail-Boucherie et al., 2002).

We conclude that the hypersensitivity to nicotine-induced seizures in Tg*CHRNA5/A3/B4* mice is explained by overexpression of nAChRs receptors that would not enhance glutamatergic neurotransmission, but instead would lead to aberrant NMDA neural transmission. Indeed, antagonists of the glutamatergic system (MK-801 and CNQX) are not able to block hypersensitivity to nicotine-induced seizures in Tg*CHRNA5/A3/B4* mice, but instead, MK-801 increases specific nicotine-induced effects in Tg*CHRNA5/A3/B4* mice.

Another interesting possibility would be that the overexpression of the cluster would affect inhibitory (GABAergic) transmission. Importantly, hippocampal α3β4* nAChRs are found on glutamatergic terminals that synapse onto interneurons and to a less extent onto excitatory pyramidal cells (Alkondon et al., 2011). We found increased c-Fos positive nuclei in CA1, SO and SR and which mainly contain GABAergic basket interneurons (Dani and Bertrand, 2007). Thus, overexpression of α3β4* nAChRs acting through the inhibitory GABAergic system could mediate the increased seizures induced by nicotine in transgenic mice. Even though at a first glance, it would be assumed that overexpression of nAChRs located on GABAergic neurons might result in enhanced inhibition, rather than increased excitability and seizures, Dobelis (Dobelis et al., 2003) already observed that not only nAChRs agonist but also nAChRs antagonists are able to elicit seizures. Therefore, decreased nicotinic function is also pro-convulsive. As a mechanism, they proposed that nAChRs located on GABAergic neurons could be responsible for nicotine-induced seizures through agonist induced desensitization of nicotinic receptors located on GABAergic neurons, resulting in net excitation. However, the fact that antagonism of α3β4* nAChRs with mecamylamine is able to smooth the

seizure phenotype in Tg*CHRNA5/A3/B4* mice, does no hold the hypothesis of desensitization as the mechanism underlying seizures.

A more likely possibility would be that activation of nAChRs on inhibitory terminals may result in enhanced GABA release that subsequently inhibits other inhibitory pathways that synapse onto excitatory neurons, overall resulting in a net excitation, and as consequence seizures (Disinhibition model) (Alkondon and Albuquerque, 2001; Freund et al., 1988). This could be thus the most likely explanation for the increased sensitivity to nicotine detected in mice overexpressing the *CHRNA5/A3/B4* cluster. Indeed, α3β4* nAChRs have been proposed to exert a powerful control on hippocampal excitability through their localization on GABAergic population (Alkondon et al., 2011).

The connection between the *CHRNA5/A3/B4* cluster and GABAergic system was also confirmed by the slightly sensitivity to PTZ, an antagonist of the GABARs that also produces clonic/tonic seizures, observed in Tg*CHRNA5/A3/B4* mice (Bastlund et al., 2005; Mandhane et al., 2007). Intriguingly, null mice for the α4 subunit of the nAChRs show normal sensitivity to nicotine-induced seizures but are highly sensitive to pro-convulsive agents that block GABA receptors, such as PTZ (Wong et al., 2003). We conclude that a deregulation of nAChRs function within the hippocampus may alter normal GABAergic neurotransmission.

Overall, the data obtained suggest that overexpression of *CHRNA5/A3/B4* cluster results in increased sensitivity to the acute pharmacological effects of nicotine. The major finding of this part of the thesis is that overexpression of the *CHRNA5/A3/B4* cluster leads to a higher activation of specific brain regions upon nicotine administration, such as the hippocampus and the piriform cortex, but not the MHb. Our results demonstrate that overexpression of the *CHRNA5/A3/B4* cluster also alters normal GABAergic neurotransmission. Hippocampal GABAergic interneurons contribute to the setting of the cooperative temporal framework that provides the basis for high cognitive functions (Rezvani and Levin, 2001). Therefore, abnormal GABAergic signalling due to overexpression of the *CHRNA5/A3/B4* may also have profound consequences on hippocampal function (see below).

5.2. CHARACTERIZATION OF THE COGNITIVE PROFILE IN Tg*CHRNA5/A3/B4* MICE

The drug addiction process shares many commonalities with normal learning and memory. Evidence supports that addictive drugs subvert normal mechanisms of neuronal adaptation, learning and memory, leading to long-lasting changes in behaviour that accrue with the ongoing progression of addiction (Dani and Harris, 2005; Dani and Heinemann, 1996; Kauer and Malenka, 2007; Kelley, 2004; Mansvelder and McGehee, 2000; Mansvelder and McGehee, 2002; Robinson and Kolb, 2004). Events and processes linked to the acquisition and delivery of drugs (for example, the environment during drug taking) become learned associations. Over time, these associations become salient cues that prompt continued drug use. Moreover, some heritable personality traits such as differences in cognitive performance are vulnerability factors in mediating smoking-related behaviour. These factors include deficits in working memory/attention, behavioural inhibition and cognitive flexibility (Janet Audrain-McGovern, 2009). In fact, tobacco use is most highly prevalent and more intense in human populations with psychiatric disorders, which use to manifest deficits in the mentioned cognitive domains (Mihailescu and Drucker-Colin, 2000). For these individuals, it is commonly argued that tobacco use may reflect attempts at self-medication (Dani and Harris, 2005). Nicotine and nicotinic agonists have long been known to improve certain types of memory-related behaviour both in humans and in a variety of animal models (Levin, 1992; Stolerman et al., 1995), and may thus serve as a positive reinforcer by facilitating cognitive performance (Benowitz, 2010; Herman and Sofuoglu, 2010; Levin et al., 2001; Levin et al., 1999; Rezvani and Levin, 2001). On the other hand, with repeated nicotine exposure, specific moods, situations, or environmental factors become associated to the reinforcing properties of the drug. These cue and contextual associations may represent a particular type of learning that contributes to craving and further cigarette smoking (Benowitz, 2010). One of the main objectives of this Thesis has been to understand how the overexpression of the cluster *CHRNA5/A3/B4* affected different aspects of cognitive performance associated to nicotine addiction and that may facilitate later on drug craving and relapse. Furthermore, we addressed the question of how the administration of nicotine either acutely or chronically modulated these cognitive capabilities.

Our experiments indicate that the impact of *CHRNA5/A3/B4* overexpression is diverse on different cognitive domains. It results in a substantial impairment in the novel object discrimination and recurrent behaviour tasks, suggesting that overexpression of the cluster *CHRNA5/A3/B4* produces deficits in working memory/attention and/or increased perseverant behaviour (also reported in Vinals et al., 2011). Importantly, acute nicotine administration completely restored the deficit in novelty discrimination observed in mice overexpressing the cluster *CHRNA5/A3/B4*, at doses with no effect in WT animals. The same tendency was observed in WT and Tg*CHRNA5/A3/B4* mice receiving chronic nicotine infusion.

Both the hippocampus and perirhinal cortex have been implicated in recognition memory (Barker et al., 2007). Furthermore, ACh plays a clear role in the modulation of both hippocampal and perirhinal-dependent learning and memory, and in novel object discrimination (Winters et al., 2006). The levels of ACh and thus, cholinergic activation influence encoding of novel information (Hasselmo and Barkai, 1995) and several studies indicate that acute systemic delivery of nAChRs agonist (Buccafusco and Jackson, 1991; Melichercik et al., 2012; Obinu et al., 2002; Puma et al., 1999) or direct nicotine infusion either into the hippocampus or perirhinal cortex enhances object discrimination memory (Melichercik et al., 2012), that we could not detect in our nicotine-treated WT group. However, discrepancies among laboratories also exist, since a recent study indicated that acute nicotine in fact impairs object recognition memory in mice, and that chronic nicotine and withdrawal from chronic nicotine had no effect (Kenney et al., 2012a). Taken together, all these studies strongly indicate that a balanced cholinergic system is required for proper novel object discrimination.

Our results suggest that overexpression of the cluster *CHRNA5/A3/B4* may alter the normal cholinergic function within the hippocampus or piriform cortex, being detrimental on the encoding of novel information. Administration of nicotine may restore the abnormal cholinergic balance in Tg*CHRNA5/A3/B4* mice and therefore ameliorate this cognitive task. Interestingly, a recent study (Winterer et al., 2010) found a significant association between single nucleotide polymorphisms (SNPs) from the *CHRNA5/A3/B4* locus and several cognitive tasks requiring discriminative abilities in humans. The authors indicated that those alleles that are associated with lower cognitive performance values are associated with increased risk for development of nicotine addiction. Moreover, the same study showed that carriers of the nicotine addiction risk

allele within the cluster *CHRNA5/A3/B4* present difficulties in working memory and attention system, and in maintaining and updating information over short delays (Winterer et al., 2010). Similarly, another group (Rigbi et al., 2008) found significant associations between SNPs in the *CHRNA5* gene and lower performance on a cognitive test that measures sustained attention and vigilance, with immediate face recognition. All these human studies are in accordance with the findings we have obtained in mice overexpressing the cluster *CHRNA5/A3/B4*. Our results, together with the data obtained in human studies suggest that the cognitive changes derived from genetic variations in the *CHRNA5/A3/B4* cluster may indirectly increase liability to nicotine addiction.

Substantial evidences demonstrate a positive correlation between development of drug addiction and deficit in behavioural inhibition and also recent memory (Janet Audrain-McGovern, 2009). Thus, we next investigated these aspects by using the passive avoidance paradigm, which measures recent memory in a task requiring behavioural inhibition. We found substantial impairment in these tasks in mice overexpressing the cluster *CHRNA5/A3/B4*. These results seem to contradict our previous work that showed reduced impulsivity in Tg*CHRNA5/A3/B4* mice using an operant delayed alternation task (Vinals et al., 2011). However, in the delayed alternation task, mice are trained to continuously change their response strategy in order to obtain food reinforcement (Weiss et al., 2005). Thus, mice must sustain attention on a previously emitted response and inhibit premature responding during the delay requirement (response inhibition). Premature responding during the delay task is associated with disruptions in the control of response inhibition and provides a measure of impulsivity (Lambourne et al., 2007; Robbins, 2002). Tg*CHRNA5/A3/B4* mice presented decreased premature responses in this paradigm, which suggests improved control of impulsivity (Vinals et al., 2011).

The discrepant results obtained in the passive avoidance paradigm that indicated reduced behavioural inhibition or altered recent memory, may thus be attributed to differences in the motivational component of both tasks. Additionally, the operant delayed alternation task is highly dependent on PFC functionality (Goldman-Rakic, 1995; Granon et al., 1994), whereas other brain regions such as the hippocampus and the amygdala, are more important in sustaining behavioural inhibition in the passive avoidance test (Izquierdo et al., 2002).

Nicotine did not reverse the deficit in the passive avoidance task observed in Tg*CHRNA5/A3/B4* mice and caused a significant impairment in control mice that remained even one week after the training session. These results contradict previous data in the literature, which indicate that nicotine ameliorates the performance of passive avoidance task (Barros et al., 2004; Nasehi et al., 2011; Nitta et al., 1994; Riekkinen et al., 1993).

Our results suggest that overexpression of the cluster *CHRNA5/A3/B4* leads to alterations in either behavioural inhibition, a personality trait directly related to drug addiction vulnerability or recent memory, and that this phenotype is not reversed by nicotine administration, thus probably being dependent on indirect or developmental effects of the cluster overexpression. Interestingly, Rigbi (Rigbi et al., 2008) demonstrated an association between genetic variability within the *CHRNA5* gene and response inhibition, and the same authors suggested that this personality trait predispose individuals to initiate and maintain cigarette smoking (Yakir et al., 2007).

We then used the Morris water maze (MWM) test to explore whether overexpression of the cluster *CHRNA5/A3/B4* resulted in alteration in other hippocampal-dependent cognitive abilities, but also on cognitive flexibility, that is dependent on the integrity of the PFC. We found that mice overexpressing the cluster *CHRNA5/A3/B4* showed improved acquisition of visuospatial learning and memory, which was significantly reversed by nicotine administration. In the probe session, Tg*CHRNA5/A3/B4* mice continued searching for the platform, thus confirming their increased reference memory. However, overexpression of the cluster *CHRNA5/A3/B4* or nicotine administration did not produce any effect regarding cognitive flexibility. Nicotine did not modify the behaviour of control animals in visuospatial learning and memory.

Spatial information processing highly depends on the hippocampus but also on a large number of cortical and sub-cortical structures (Deiana et al., 2011). Cholinergic afferents from the medial septal nucleus control encoding, consolidation and retrieval of visuospatial learning (Hasselmo and Barkai, 1995). Blockade of nAChRs leads to spatial memory deficits while their activation of nAChRs by nicotine has been reported to improve spatial learning (Abdulla et al., 1996; Abdulla et al., 1993; Deiana et al., 2011; Hodges et al., 1995; Riekkinen and Riekkinen, 1997; Riekkinen et al., 1996). However, some studies have indicated that infusion of nicotine evokes a modest

impairment of the acquisition of spatial learning task (Bernal et al., 1999; Scerri et al., 2006), or had no effect (Addy et al., 2003). The genetic background has a strong contribution on the effects of nicotine on spatial learning and memory. Actually, the agonist for the β4 subunit SIB-1553A have been shown to improve retention of reference memory in water maze in C57BL/6 mice while it impairs retention of reference memory in DBA/2 mice (Sunyer et al., 2008). This data supports the involvement of the β4 subunit in spatial memory and the idea that genetic variability among individuals or populations may account for profound differences in the behavioural responses to nicotine. In human studies, Zhang (Zhang et al., 2010) confirmed that variants within the gene cluster *CHRNA5/A3/B4* modulated cognitive flexibility, in a population specific manner. In European Americans five markers within the cluster *CHRNA5/A3/B4* were associated with less cognitive flexibility, i.e. more perseverative responses and perseverative errors. Conversely, in African American population markers in the *CHRNA5* gene were associated with reduced perseverative errors. The authors concluded that genetic variability within the cluster *CHRNA5/A3/B4* may affect cognitive flexibility differentially depending on genetic background. In our model, the overexpression of the cluster *CHRNA5/A3/B4* improved high order cognitive performance, thus enhancing goal-directed learning, which may have consequences on acquisition of maladaptive behaviours that subserve drug addiction.

Finally, we aimed at investigating whether overexpression of the cluster *CHRNA5/A3/B4* altered contextual associations. Repeated drug use in particular environments and contexts causes a strong but maladaptive association between the effects of the drug and contextual stimuli. The fear conditioning paradigm is an attractive model for studying the neural bases of learning and memory associated to contextual information (Davis and Gould, 2008; Davis et al., 2006; Gould et al., 2004; Gould and Wehner, 1999; Kenney and Gould, 2008a; Kenney and Gould, 2008b). We used this paradigm in order to investigate if deregulation of the expression of the α5, α3 and β4 subunits of the nAChRs would facilitate contextual fear conditioning. Contrary to our hypothesis, we found that overexpression of the cluster *CHRNA5/A3/B4* did not modify neither acquisition nor memory for contextual fear conditioning. However, our results are in accordance with previous data demonstrating that null mice for the β4 subunit show similar behaviour in the contextual fear conditioning paradigm compared to their WT littermates (Wehner et al., 2004).

We also investigated whether chronic nicotine treatment strengthened contextual associations more significantly in mice overexpressing the cluster *CHRNA5/A3/B4*. Several lines of evidence suggest that nicotine enhances hippocampus-dependent (i.e. contextual) fear conditioning (Davis and Gould, 2008; Davis et al., 2006; Gould et al., 2004; Gould and Wehner, 1999; Kenney and Gould, 2008a; Kenney and Gould, 2008b) when administered acutely but not chronically (Andre et al., 2008). Surprisingly, we found that nicotine treatment produced opposite effects on acquisition learning depending on the genotype. In WT mice, nicotine enhanced acquisition learning of contextual fear conditioning, while in Tg*CHRNA5/A3/B4* mice it produced deleterious effects, but had no consequences on memory the testing day. Learning to associate the context with the US stimulus is both hippocampus and amygdala dependent (Kim et al., 1993; Maren, 2001). Our results suggest that the cluster *CHRNA5/A3/B4* may not be involved in the formation of contextual associations that contribute to nicotine addiction and relapse. Normally, when exposed to smoking vs. neutral images smokers exhibit greater brain reactivity in regions involved in reward and learning processes (Janes et al., 2011). Interestingly, smokers carrying a risk allele for nicotine addiction in *CHRNA5* gene (D398N) show reduced functional magnetic resonance imaging (fMRI) reactivity to smoking-related vs. neutral images with reduced activation of several limbic regions, including cingulated cortex, hippocampus and amygdala (Hong et al., 2010).

In summary, our results indicate that overexpression of the *CHRNA5/A3/B4* cluster leads to deficits in a number of specific cognitive domains associated to nicotine addiction. It is conceivable that individual genetic variability in the clustered genes may be a determinant factor for initiation and maintenance of addiction to nicotine in order to compensate specific cognitive alterations.

5.3. CHARACTERIZATION OF THE HIPPOCAMPUS IN Tg*CHRNA5/A3/B4* MICE

The key elements of nicotinic action on cognition may be linked to the hippocampus, an important neuroanatomical structure for learning and memory processes. Previous results in our laboratory indicated that Tg*CHRNA5/A3/B4* mice have increased nicotine binding sites (Gallego et al., 2012) mainly constituted by

increased α3β4* nAChRs in the hippocampus, principally in the CA1 subregion (Gallego et al., 2011). From the behavioural point of view, Tg*CHRNA5/A3/B4* mice show deficits in hippocampal-dependent cognitive domains, such as the novel object discrimination task. Additionally, we found that hypersensitivity to nicotine-induced seizures observed in Tg*CHRNA5/A3/B4* mice resulted from increased activation of the hippocampus, particularly the CA1 subregion. Since nicotine, like other psychostimulant drugs, induces substantial neuroplastic changes in the brain (Robinson and Kolb, 2004; Tang and Dani, 2009), at the level of structure (structural plasticity) or synaptic strength (synaptic plasticity), these data suggested that overexpression of the *CHRNA5/A3/B4* cluster could have consequences on the structure and function of the hippocampus. Structural plasticity refers to changes in neuronal morphology (including axons, dendrites and dendritic spines); whilst synaptic plasticity refers to biochemical modifications related to changes in synaptic activity, and may underlie maladaptive behaviours such as those involved in drug addiction. Thus, we also wanted to elucidate whether overexpression of the *CHRNA5/A3/B4* cluster facilitates the aforementioned neuroplastic changes induced by nicotine.

We first demonstrated that overexpression of the cluster *CHRNA5/A3/B4 per se* did not produce significant abnormalities in the general morphology of the hippocampus using *Thy1-YFP* double transgenic/WT mice. This data is in accordance with previous results, which showed that α5 nAChRs null mice display normal hippocampal morphology (Salas et al., 2003). However, we found that chronic nicotine treatment, affected differentially the morphology of the CA1 pyramidal layer of the hippocampus, depending on the genotype. Surprisingly, while in WT mice, chronic nicotine increased the length of CA1 pyramidal layer, it reduced the length of this layer in Tg*CHRNA5/A3/B4* mice. Previous data indicated that 6 days of chronic nicotine treatment is enough to produce modifications in brain regions involved in reward, such as the VTA (Bunnemann et al., 2000). However, we are the first examining the effects of chronic nicotine treatment in the morphology of adult hippocampus. Several studies have shown that abnormal cholinergic signalling during periods of development produced by smoking during pregnancy, results in altered morphological features in hippocampus and cortex (Huang et al., 2007; Roy and Sabherwal, 1994; Roy and Sabherwal, 1998; Roy et al., 2002) that may contribute to alterations in cognitive performance later in live (Huang et al., 2007). Our results suggest that genetic

variability in the cluster *CHRNA5/A3/B4* account for differences in structural plasticity produced by nicotine. This increased sensitivity to neuroadaptations produced by nicotine in mice overexpressing the cluster *CHRNA5/A3/B4* may be the cause of differential behavioural responses we have been previously demonstrated.

The transition from occasional drug use to addiction involves neuroplasticity mechanisms in brain regions involved in the development of addiction (Koob and Le Moal, 2001). To further investigate the effects of nicotine on structural plasticity we used hippocampal primary cultures. We found that neurons derived from hippocampal cultures overexpressing the cluster *CHRNA5/A3/B4* showed significantly reduced arborisation compared to neurons from control mice. Nicotine exposure during 48 hours reduced branching complexity in WT neurons but instead completely restored arborisation in transgenic neurons. This result is surprising, since psychostimulant drugs, including amphetamine, nicotine, and cocaine, lead to increases in dendritic arborisation and spine density in brain regions involved in both reward and learning phenomena, such as the NAc and prefrontal cortex (Brown and Kolb, 2001; Li et al., 2003; Miyata and Yanagita, 2001; Pich et al., 1998; Robinson and Kolb, 1997; Robinson and Kolb, 2004; Tang and Dani, 2009). Nicotine binding to presynaptic nAChRs facilitates the release of excitatory neurotransmitters and also GABA (Wonnacott, 1997; Wonnacott et al., 1990), causing a direct effect on neuronal activity. Activation of nAChRs either directly (Girod et al., 2000; McGehee and Role, 1996) or indirectly, through VGCC or release of calcium from intracellular stores (Chang and Berg, 2001; Tsuneki et al., 2000) increases intracellular calcium levels, activating downstream signalling cascades, such as Akt, PI3K, Ras-ERK, cAMP-PKA-CREB, implicated in the structural plasticity that occurs in brain after drug administration (Reviewed in (Dietz et al., 2009)). For example, it has been reported that psychostimulants produce widespread, but transient induction of BDNF in brain regions involved in reward (Graham et al., 2007; Grimm et al., 2003) providing a possible mechanism for induction and maintenance of structural plasticity (Bramham and Messaoudi, 2005). In contrast, nAChRs agonists produce long lasting increases of other neurotrophic signalling molecules, such as nerve growth factor (NGF) or fibroblast growth factor 2 (FGF), that could mediate structural plasticity (Buccafusco et al., 2005) through calcium-dependent activation of CaMKII and the ERKs (Ha and Redmond, 2008; Vaillant et al., 2002). Interestingly, NGF treatment has been demonstrated to

increase mRNA levels of the α3 (Yeh et al., 2001) and the β4 (Hu et al., 1994) subunits of the nAChRs, suggesting an intrinsic relationship between the neurotrophic factors that regulate dendrite formation and changes in the expression of the nAChRs subunits encoded in the cluster *CHRNA5/A3/B4*. Furthermore, nAChRs with high calcium permeability, such as those containing the α5 subunit, have special importance in calcium-dependent neuronal plasticity (Winzer-Serhan and Leslie, 1997).

Psychostimulant drugs, including nicotine, also increase the density of dendritic spines in brain regions involved in reward and learning processes (Brown and Kolb, 2001; Li et al., 2003; Robinson and Kolb, 1997). In our experiments, neurons derived from transgenic hippocampal cultures presented increased filopodia-like and type III (thin) spines along with reduced density of type I (stubby) spines, as compared to WT neurons. Interestingly, the same phenotype was observed in neurons derived from WT mice treated with nicotine. Thus, both overexpression of the cluster *CHRNA5/A3/B4* and nicotine treatment increase the density of filopodia-like structures. Previous studies have shown that increased nicotinic transmission results in higher number of filopodia-like structures (Kawai et al., 2002), an effect mediated by nAChRs containing the α7 subunit, which are found sharply concentrated at the distal tips of filopodia-like extensions (Kawai et al., 2002; Lippi et al., 2011). In a recent study, the authors found that this increase in filopodia-like structures may result from altered expression of specific miR, which contribute to the plastic changes underlying addictive behaviours (Chandrasekar and Dreyer, 2009; Hollander et al., 2010).

In our experiments, the increase of filopodia was not correlated with increased structural complexity of the neurons. However, recent studies propose that it is the filopodia stability but not the number, what leads to more axonal-dendritic contacts (Arstikaitis et al., 2011). Moreover, increases in filopodia-like structures can also be consequence of reduced synaptic plasticity, reflecting an attempt to compensate for the loss of synaptic input (Kirov and Harris, 1999). Because α3β4* nAChRs predominate on GABAergic hippocampal neurons it may be possible that the overexpression of the *CHRNA5/A3/B4* cluster reduced the neuronal activity of the hippocampus, that is compensated by increasing the density of filopodia-like structure.

Changes in the morphology of dendritic spines have a close relationship with the strength of its synaptic connection, as measured as the ability of the synapse to undergo long-term potentiation (LTP), one of the major cellular mechanisms underlying learning

and memory (Yuste and Bonhoeffer, 2001). The altered the morphology of dendritic spines, towards increasing the density of thinner and immature spines driven by overexpression of the *CHRNA5/A3/B4* cluster may thus have consequences on synaptic strength.

To address this question, extracellular field excitatory postsynaptic potentials (fEPSPs) evoked by Schaffer collateral stimulation were recorded from stratum radiatum of the dorsal CA1 hippocampal region in slices from control and Tg*CHRNA5/A3/B4* mice. Two different synaptic phenomena were evaluated, paired pulse facilitation ratio (PPR) and LTP. Synaptic facilitation is a form of short-term synaptic plasticity that has been observed at many chemical synapses and has been associated with increases in presynaptic calcium levels and neurotransmitter release (Zucker, 1989). PPR is expressed in electrophysiological experiments as changes in the amplitude/slope of a test EPSP evoked by a second presynaptic spike that follows the first one (Zucker, 1989). We found similar increased synaptic efficacy upon paired stimulation in slices from WT and Tg*CHNA5/A3/B4* mice. However, nicotine increased the magnitude of the PPR more markedly in Tg*CHNA5/A3/B4* mice, suggesting that the probability of glutamate release from the Schaffer collateral was increased when hippocampus was exposed to nicotine more significantly in Tg*CHNA5/A3/B4* mice and may explain the nicotine-enhancing cognitive effects that we have observed in behavioral tasks depending on the dorsal hippocampus, such as novel object discrimination.

We also analyzed LTP upon HFS application to SC pathway. We found that HFS elicited a significant and stable LTP in slices from WT mice but it evoked a reduced potentiation in hippocampal slices from Tg*CHRNA5/A3/B4* mice. Several studies have demonstrated that ACh modulates LTP expression in the CA1 subregion of the hippocampus (Freir and Herron, 2003; Fujii et al., 1999; Fujii et al., 2000; Hunter et al., 1994). At most synapses, LTP is triggered by postsynaptic calcium influx through NMDARs (Herron et al., 1986), and under some conditions, through L-type VGCC (Grover et al., 2009). A possible mechanism explaining the reduced potentiation in Tg*CHRNA5/A3/B4* mice after HFS may be that endogenous ACh increased activation of $\alpha3\beta4$* nAChRs located on GABAergic interneurons that directly synapse on pyramidal neurons, thus exerting a powerful inhibition in hippocampal neurocircuitry and impeding LTP occurrence. Indeed, acting primarily on presynaptic $\alpha3\beta4$ nAChRs, ACh

depolarizes GABAergic axon terminals enough to open T-type calcium channels. The resulting calcium influx triggers GABA release that can transiently reduce pyramidal cell excitability (Tang et al., 2012).

Evidence accumulated that nicotine is able to shift short-term potentiation (STP) to long-term potentiation (LTP)(Drever et al., 2011; Kauer and Malenka, 2007; Mao et al., 2011). Also, since nicotine administration reversed some of the abnormal behavioral and structural alterations in Tg*CHRNA5/A3/B4* animals, we investigated whether it was possible to rescue LTP in Tg*CHRNA5/A3/B4* mice by treatment with nicotine. We found that nicotine exposure enhanced LTP in WT mice when HFS was applied, confirming previous findings that nicotine is able to enhance LTP in SC-CA1 pathway in control mice (Fujii et al., 1999). However, it had no effect on HFS-induced LTP in Tg*CHRNA5/A3/B4* mice.

Nicotine might facilitate LTP induction via activation of GABAergic nAChRs that increase activity in CA1 pyramidal cells, presumably via inhibiting other inhibitory interneurons (Ji et al., 2001) or alternatively, a decrease in inhibitory interneuron activity following nAChRs desensitization (Fujii et al., 2000; Nakauchi and Sumikawa, 2012; Yamazaki et al., 2005). Additionally, nicotine acting on postsynaptic nAChRs at CA1 pyramidal cells reduces the threshold for LTP induction in the SC-CA1 pathway (Ji et al., 2001). Either the two firsts mechanisms might be altered when the *CHRNA5/A3/B4* cluster is overexpressed, because of the localisation of $\alpha3\beta4$* nAChRs in GABAergic interneurons and the increased rate of desensitization upon incorporation of the $\alpha5$ subunit.

The present results suggest that changes in the expression of the $\alpha5$, $\alpha3$ and $\beta4$ subunits of the nAChRs are an important factor modulating the functionality of the hippocampus. A deregulation in the expression of these subunits leads to reduced complexity of hippocampal neurons along with increased density of immature spines. A close relationship exists between neuronal morphology and synaptic efficiently. Our results pointed that overexpression of the cluster *CHRNA5/A3/B4* causes an unbalance between excitatory and inhibitory neurotransmission towards enhanced GABAergic neurotransmission in the hippocampus yielding a net effect on neuronal morphology and synaptic plasticity.

Interestingly, several studies suggest that chronic nicotine increases nAChRs number in the dorsal but not ventral hippocampus (Abdulla et al., 1996), but the

consequences of nAChRs receptor upregulation on structure and function of the hippocampus have not been investigated. A recent study suggests that the combined upregulation and desensitization that occurs while nicotine is present may help to maintain a functional homeostatic state for the cholinergic system within the hippocampus to support proper cognitive function (Gould et al., 2012). Once nicotine is no longer present (i.e. nicotine withdrawal), these changes could result in a hyper-sensitive nACh system, leading to cognitive deficits. Indeed, nicotine withdrawal was associated with a persistent increase in hippocampal CA1 pyramidal activity and with cognitive deficits (Kenney et al., 2012b; Penton et al., 2011).

Noticeably, the transcriptional regulation of the α5, α3 and β4 nAChRs subunits is under Ras-dependent MAPK and PI3K and MEK activated pathways (Melnikova and Gardner, 2001) which are principal intracellular signalling cascades in learning and reward processes. The fact that the expression of the α3, β4 and α5 subunits is under regulation of synaptic signalling further supports the idea that these subunits play important roles in neuronal plasticity. Taken together our results indicate that the overexpression of α5, α3 and β4 nAChRs subunits leads to a complex cognitive profile with specific dorsal hippocampal deficits at the behavioural and functional levels. The fact that nicotine restores neuritogenesis deficits in primary hippocampal cultures of Tg*CHRNA5/A3/B4* mice, along with the slight structural hippocampal changes observed upon nicotine treatment in vivo, suggest that neural plasticity changes in brain areas involved in learning and memory are important mechanisms involved in the genetic susceptibility to nicotine addiction in Tg*CHRNA5/A3/B4* mice.

5.4. CHARACTERIZATION OF THE NICOTINE ADDICTIVE PROFILE IN Tg*CHRNA5/A3/B4* MICE

The last part of the Thesis has been focused on studying how the overexpression of the cluster *CHRNA5/A3/B4* affected several aspects of nicotine addiction, such as nicotine self-administration and the development of tolerance and withdrawal.

There are several animal models to study nicotine self-administration; each of them with more or less powerful face, predictive and construct validity (reviewed in O'Dell and Khroyan, 2009). One of these models consists of supplying nicotine in drinking water (Rowell et al., 1983). Previous results in the laboratory demonstrated

increased reinforcing effects of nicotine in Tg*CHRNA5/A3/B4* mice, in a simple fixed-ratio schedule of intravenous nicotine self-administration (IVSA) (Gallego et al., 2011). In this work, and in view that Tg*CHRNA5/A3/B4* mice showed cognitive differences, and IVSA involves learning of an operant behaviour, we decided to use the two bottle choice paradigm in order to investigate whether overexpression of the cluster *CHRNA5/A3/B4* alter nicotine self-administration when no learning aspects are involved.

At very low doses of nicotine none of the genotypes showed preference for nicotine-containing solutions over control, according to previous results (Meliska et al., 1995; Robinson et al., 1996). As the concentration of nicotine increased there was a general decrease in nicotine preference in both genotypes, reflecting development of aversion to nicotine. However, overexpression of the *CHRNA5/A3/B4* cluster resulted in significantly higher aversion to nicotine at high doses of the drug (200 μg/ml) compared to WT. The negative effects of the drug were not due to the taste properties of the drug, but instead, to the development of aversive effects of nicotine intake that were accentuated when overexpression of the *CHRNA5/A3/B4* cluster.

To understand the discrepancy of these results to those obtained using the IVSA protocol we have to bear in mind that, although both paradigms evaluate nicotine self-administration, substantial differences exist among them. First, IVSA mice have limited access to the drug for 2 hours per day, while in the two-bottle choice paradigm they have 24 hours access, and several studies have indicated that limited access to the drug can induce high drug intakes in short periods of time (Koob and Le Moal, 2005). Second, even though it is generally argued that the IVSA paradigm assesses the rewarding properties of an addictive drug, it should also be considered that this task involves the learning of having to perform an operant behaviour. Thus, it may be possible that Tg*CHRNA5/A3/B4* mice present an earlier acquisition of nicotine IVSA, since we have observed that nicotine facilitates specific types of learning in Tg*CHRNA5/A3/B4* mice. A recent study suggests that α5 subunits are critical for the regulation of DA transmission by α4β2* nAChRs in regions of striatum associated with habitual and instrumental responses (dorsal CPu) rather than pavlovian associations (NAc) (Exley et al., 2012). Overexpression of the cluster *CHRNA5/A3/B4* may thus specifically facilitate instrumental associated to addiction to nicotine but not other types of learning (see above).

Overexpression of the *CHRNA5/A3/B4* cluster results both in higher sensitivity to nicotine-induced seizures and also higher aversion to nicotine intake. The MHb is involved in the aversive properties of nicotine and has a major influence on the control of nicotine self-administration (Fowler et al., 2011; Frahm et al., 2011; Glick et al., 2002). Approximately 90–100% of neurons located in the MHb express α3, α4, α5, β2 and β4 nAChR subunits (Tuesta et al., 2011). The important role of α5* and β4* nAChRs in regulating drug self-administration came from studies showing that 18-methoxycoronaridine (18-MC) (Vastag, 2005), a potent antagonist of β4* nAChRs (Glick et al., 2002) has significant anti-addictive properties (Maisonneuve and Glick, 2003). In animal models, 18-MC injected to the MHb reduced self-administration of nicotine, morphine, cocaine and methamphetamine, and oral intake of alcohol (Maisonneuve and Glick, 2003; Taraschenko et al., 2007). Moreover, null mice for the α5 subunit show increased self-administration of nicotine, especially at high doses of the drug (Fowler et al., 2011; Jackson et al., 2010). Furthermore, the balanced activity between the α5 and β4 subunits of the nAChRs in the MHb controls nicotine intake through regulation of the aversive properties of the drug (Frahm et al., 2011). Consistent with these studies our results confirm that a deregulation in the expression of the α3, α5 and β4 subunits alter the pattern of nicotine self-administration, since Tg*CHRNA5/A3/B4* mice show increased α3β4* nAChRs in the MHb (Gallego et al., 2011). However, c-Fos experiments showed acute nicotine injection increased the number of c-Fos positive nuclei but with no significant differences between genotypes. Also, activation of VTA DAergic neurons was significantly reduced in Tg*CHRNA5/A3/B4* mice upon acute nicotine administration. Since the dose of nicotine used in these experiments was very low these results are in line with the lack of significant differences in self-administration at low nicotine doses (see above). On the other hand, the rates of nicotine consumption when it is presented at high nicotine concentration shows a significant correlation with the number of nAChRs binding sites in the hippocampus and cortex (Li et al., 2005) and Tg*CHRNA5/A3/B4* mice show a substantial increase in the expression of α3β4*-nAChRs in the CA1 region of hippocampus.

Interestingly, pharmacological studies have also implicated α3β4* nAChR in ethanol consumption and seeking in rats (Chatterjee et al., 2011) as well as in alcohol and nicotine co-dependencies. Studies with nAChRs mutant mice (Butt et al., 2003;

Owens et al., 2003) support a common physiological basis for alcohol and nicotine's behavioural effects and these effects may converge on the cluster *CHRNA5/A3/B4*. As occurred with nicotine in the present work, previous results in our laboratory indicate that Tg*CHRNA5/A3/B4* mice consumed less amount of alcohol when available in drinking water (Gallego et al., 2012), showing a similar pattern to that we observed for nicotine self-administration. The results confirm the existence of a considerable genetic influence on both alcoholism and smoking and the involvement of the α5, α3 and β4 subunits of the nAChRs in such phenotypes.

We also tested whether pre-exposing mice to a high dose of nicotine could modify the pattern of nicotine self-administration. A single exposure to a high dose of nicotine increased the aversion to high doses of nicotine in Tg*CHRNA5/A3/B4* animals (50 and 200 μg/ml). It may be possible that the first exposure to nicotine may have produced consequences on the central reward circuits thus modifying the subsequent nicotine consumption. Indeed, a recent study has shown that pre-exposure to pharmacological agents that act upon reward circuits significantly changes the pattern of nicotine self-administration in the two-bottle choice paradigm, possibly through a direct change in the central reward circuits modifying the sensitivity to the rewarding effects of nicotine (Cao et al., 2012). According to these results, the overexpression of the cluster *CHRNA5/A3/B4* may favour these plastic changes. Also, sensitivity to nicotine intake shows a highly correlation with nicotine-induced seizures (Robinson et al., 1996). Both findings totally fit with the results we obtained in our study, since overexpressing the cluster *CHRNA5/A3/B4* results both in higher sensitivity to nicotine-induced seizures and also higher aversion to nicotine intake.

Prolonged nicotine use triggers neuroadaptations that contribute to the development of dependence, tolerance and reinforcement of nicotine use (Laviolette and van der Kooy, 2004). Chronic nicotine administration has been shown to induce physical dependence and nicotine induced tolerance. We aimed to investigate how overexpression of the cluster *CHRNA5/A3/B4* affected nicotine withdrawal and tolerance. We found that Tg*CHRNA5/A3/B4* mice exhibited significantly greater number of somatic signs of withdrawal compared to nicotine-treated WT mice only when withdrawal was spontaneously induced but not precipitated with mecamylamine. The dose and treatment duration were possibly not sufficient to induce changes in affective signs of withdrawal or hyperalgesia, since Damaj (Damaj et al., 2003) reported

that in mice the dose of nicotine and duration of chronic infusion necessary to induce hyperalgesia and anxiety-like behaviour was at least 24 mg/kg/d for 14 days.

Nicotine withdrawal signs seem to be mediated by multiple nAChRs. Both the β4 and α5 nAChRs subunit had been found to be involved in the somatic sings (Jackson et al., 2008; Salas et al., 2004b; Salas et al., 2009), but not the affective signs associated with nicotine withdrawal. Several studies indicate that the Hb-IPN system is implicated in withdrawal from drugs of addiction, including nicotine (De Biasi and Salas, 2008; Salas et al., 2009). Mecamylamine precipitates somatic signs and hyperalgesia but not anxiety like behaviour, in accordance with our results. Affective signs of nicotine withdrawal are only precipitated with the selective antagonist for the α4β2* nAChRs (Damaj et al., 2003). These results suggest that nicotine withdrawal signs are mediated by multiple neuronal nicotinic subtypes and that the dose and duration of nicotine exposure influence the development of nicotine withdrawal and severity.

Finally, overexpression of the cluster *CHRNA5/A3/B4* does not modify the development of tolerance to the effects of nicotine. Chronic infusion with nicotine results in tolerance to nicotine-induced seizures (Miner and Collins, 1988a; Miner and Collins, 1988b). We found reduced seizures in mice chronically treated with nicotine, but there were no significant differences in mice overexpressing the cluster *CHRNA5/A3/B4*. It should be noted that because of the increased sensitivity to nicotine in seizures other experiments may be required to conclude that overexpression of the cluster *CHRNA5/A3/B4* does not influence over development of tolerance to the effects of nicotine.

Here we report that overexpression of the cluster *CHRNA5/A3/B4* shifts the sensitivity to the rewarding and aversive properties of nicotine. Meanwhile low doses of nicotine appear to be more reinforcing in Tg*CHRNA5/A3/B4* animals; high doses of nicotine are prone to be more aversive. Our major finding is that a single exposure to a high dose of nicotine shifted the aversive properties of nicotine exclusively in mice overexpressing the cluster *CHRNA5/A3/B4*. Furthermore, the overexpression of the three subunits increases the somatic aspect of withdrawal after cessation of chronic nicotine. Currently, there are 3 types of pharmacotherapies approved in the USA to help quit smoking: nicotine replacement therapy, varenicline and bupropion. Interestingly, all three pharmacological strategies target β4-containing receptors (Baldwin et al., 2011). People carrying genetic variant that modulate α5, α3 and β4 nAChRs will show differential levels of basal reward and disappointment (aversion) states, thereby modulation not only tobacco addiction but other addictions and reward-related behaviours in general. Furthermore, we propose that the α5, α3 and β4 subunits are responsible for the neuroadaptations that occur after prolonged exposure to nicotine, and crucial for the development of nicotine addiction. Our study provides a model for exploration of the involvement of nAChRs containing the α5, α3 and β4 subunits in nicotine consumption.

CONCLUSIONS

6. CONCLUSIONS

- The overexpression of the *CHRNA5/A3/B4* genomic cluster increases sensitivity to the acute pharmacological effects of nicotine on hypolocomotion and seizures. Changes in GABAergic neurotransmission in brain regions, such as the hippocampus and piriform cortex may contribute to this phenotype.

- The overexpression of the *CHRNA5/A3/B4* genomic cluster gives rise to a distinctive cognitive profile, with increased perseverant behaviour and impaired response inhibition and novelty recognition. Importantly, some of these cognitive alterations could be compensated upon nicotine administration.

- The overexpression of the *CHRNA5/A3/B4* genomic cluster disturbs structural and functional characteristics of the hippocampus, concerning neuronal complexity, spine morphology and synaptic plasticity. These structural and functional adjustments respond differentially to nicotine administration.

- The overexpression of the *CHRNA5/A3/B4* cluster shifts the sensitivity of the rewarding and aversive properties of nicotine and increases manifestation of withdrawal syndrome upon cessation of chronic nicotine exposure.

It is conceivable that individuals with genetic variability within the genomic cluster *CHRNA5/A3/B4* may be prone to initiate and maintain smoking-related behaviours because of specific changes in brain structure and function and due to increased sensitivity to the pharmacological effects of nicotine. The increased sensitivity to nicotine may help to compensate cognitive performance in genetically susceptible individuals, but may also facilitate acquisition of maladaptive behaviours associated to drug addiction through enhancing structural and synaptic plasticity adaptations in specific brain structures. Concretely, the hippocampus may be an important substrate of addiction in individuals with genetic variability on the clustered genes, converging on both learning and reward phenomena.

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ANNEX

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ANNEX I: SUPPLEMENTARY MATERIAL AND METHODS

a) Generation of Tg*CHRNA5/A3/B4* mice

Tg*CHRNA5/A3/B4* mice were generated in the Transgenesis Unit of the Medical and Molecular Genetics Centre (CGMM-IRO) by standard pronucleus microinjection of a 111kb BAC fragment inserted with the human *CHRNA5/A3/B4* cluster on a B6/SJL-F1J genetic background (Gallego et al., 2011). The presence of all promoter regions was assessed by PCR on maxiprep-extracted DNA from the RP11-335K5 (AC067863) clone, and rearrangements within the BAC were checked (EagI (BshTI) restriction pattern). DNA from tail biopsies was used for genotyping routinely by PCR analysis using two primer pairs: *CHRNB4* human/forward 5'- gagccaagatcccaccactc – 3' and *CHRNB4* human/reverse 5'- ccaggcatccggatttgtat 3'; *CHRNA5* human/forward 5'-gaaagacttgagtgggcagc – 3' and *CHRNA5* human/reverse 5'-caaccctgtctgtctctagc -3'. Because pronuclear injection leads to a random insertion of the transgene, two different transgenic lines were generated in order to exclude insertion positional effects, line 22 (carrying between 16-18 copies of the transgene) and line 30 (carrying between 4-5 copies of the transgene). The mouse colony was maintained by crossing transgenic mice with hybrid B6/SJL-F1 female mice (F1-F5). The non-transgenic littermates obtained from crosses of Tg*CHRNA3/A5/B4* mice and B6/SJL-F1J females (wild type, WT) served as controls. Breeding and colony management were carried out at the Barcelona Biomedical Research Park (PRBB) animal facility.

Since previous studies in the laboratory (Gallego et al., 2011) demonstrated a similar behaviour between the two transgenic lines, all experiments performed in this Thesis used exclusively the line 22. Three days before testing, each mouse was handled for 2 min/day in order to reduce stress levels that may affect posterior behaviour. For those experiments that required an injection, mice were extra-handled to being hand-restricted by the experimenter. For all experiments, animals were acclimated on the behavioural room for at least 30 min before being tested. In all the experiments, the

experimenter was the same, blind with respect to the mouse genotype or treatment and had the necessary accreditation to work with laboratory mice.

b) Minipump implantation

Mice were implanted with Alzet minipump model 2001 that infused for 7 days either saline (NaCl 0.9%) or nicotine hydrogen tartrate (10mg/kg/day, Sigma) dissolved in saline. Before filling the osmotic minipumps, the pH was adjusted to 7.4 and drug solutions were sterilised by filtration through a 22-μm filter. The minipumps were surgically implanted subcutaneously under sterile conditions with O_2 - isofluorane mixture anaesthesia. An incision was made on the back of the animal and the minipump was carefully inserted under the skin. Rapidly, the incision was stitched with sterile sewing thread and coated with antiseptic iodine-based derivate to prevent infection. The animals were individually housed to keep the wound protected. Alzet minipumps consist of three concentric layers: the inner drug reservoir, the osmotic layer and the outer controlled semipermeable membrane. The reservoir was filled with sterile physiological saline or nicotine and the concentration of nicotine was adjusted according to the animal weight resulting in 10 mg/kg/day. After the minipump was implanted, extracellular liquid was absorbed though the outer membrane and that expanded the osmotic layer compressing the impermeable flexible reservoir, and releasing the drug solution through the exit port in a concrete rate.

c) Electrophysiology in hippocampal slices

For paired-pulse facilitation and LTP recordings mice were deeply anaesthetized with urethane and after decapitation, the brain was quickly removed and placed in ice-cold cutting solution (in mM): KCl 2.5; $MgSO_4$ 3; $NaHPO_4$ 1.25; $CaCl_2$ 1; $NaHCO_3$ 26; sucrose 10 and was gassed with 95% O_2-5% CO_2 to a final pH of 7.4. Coronal slices (400 μm thick) were obtained with a vibratome and slices were placed in an interface-style recording chamber (Fine Science Tools, Foster City, CA) and bathed in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl, 124; KCl, 2.5; $MgSO_4$, 1; $NaHPO_4$, 1.25; $CaCl_2$, 2.5; $NaHCO_3$, 26; and dextrose, 10 gassed with 95% O_2-5% CO_2 to a final pH of 7.4. Bath temperature was maintained at 32–34°C. Electrical stimuli

were delivered with glass electrodes (impedance 1-2 MΩ) with the stimulus strength adjusted to twice the threshold response intensity. Extracellular, unfiltered recordings were obtained by means of glass electrodes (impedance 1-2 MΩ) through a Neurolog system (Digitimer) amplifier.

ANNEX II: ABBREVIATIONS

5HT Serotonin

18-MC 18-methoxycoronaridine

ACh Acetylcholine

nAChR Nicotinic acetylcholine receptor

ACSF Artificial cerebrospinal fluid

ACTH Adrenocorticotropic hormone

ADHD Attention deficit hyperactivity disorder

AMPA Alpha-amino-5-hydroxy-3-methyl-4-isoxazolepropionic acid

BAC Bacterial artificial chromosome

BDNF Brain-derived growth factor

CA Corpus ammonis

CaMKII Calcium-calmodulin-dependent kinase

ChAT Choline-acetyltransferase

CNQX 6-cyano-7-nitroquinoxaline-2,3-dione

CNS Central nervous system

CPu Caudate putamen

CREB cAMP response element-binding

CRH Corticotropine release hormone

CS Conditioned stimulus

D–R Dose-response

DA Dopamine

DG Dentate gyrus

DIV Day in vitro

E Embryonic day

EGFP Enhanced green fluorescence protein

fEPSPs Evoked field excitatory postsynaptic potentials

ERK Extracellular regulated kinase

GABA Gamma-aminobutyric acid

$GABA_A$ GABA Receptor type A

GCL Granular cell layer

GWAS Genome-wide association studies

Hb Habenula

HFS High frequency stimulation

IPN Interpeduncular nucleus

IVSA Intravenous self-administration

KO Knockout

LC Locus coeruleus

LDTg Laterodorsal tegmentum

LEnt Lateral entorhinal cortex

LGIC Ligand-gated ion channels

LHb Lateral habenula

LTD Long term depression

LTP Long term potentiation

MAPK Mitogen activated protein kinase

MHb Medial habenula

MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MWM Morris Water Maze

NAc Nucleus accumbens

NGF Nerve growth factor

NMDA N-methyl-D-aspartic acid

NO Nitric oxide

NPY Neuropeptide Y

PFC Prefrontal cortex

PI3K Phosphoinositide 3 kinase

PKA Protein kinase A

PKC Protein kinase C

PNS Peripheral nervous system

POB Phenoxybenzamine

PPI Prepulse inhibition

PPR Paired-pulse facilitation ratio

PPTg Pedunculopontine tegmentum

PSD Postsynaptic density

PTZ Pentylenetetrazole

SC Schaffer collateral

SO Stratum oriens

SNP Single nucleotide polymorphism

SNc Substantia nigra pars compacta

SR Stratum radiatum

STP Short-term potentiation

TBS Theta burst stimulation

TH Tyrosine hydroxylase

US Unconditioned stimulus

VGCC Voltage-gated calcium channels

VTA Ventral tegmental area

WT Wild type

YFP Yellow fluorescent protein

ANNEX III:

PUBLICATIONS

Overexpression of the CHRNA5/A3/B4 genomic cluster in mice increases the sensitivity to nicotine and modifies its reinforcing effects

Xavier Gallego, Susanna Molas, Alejandro Amador-Arjona, Michael J. Marks, Noemí Robles, Patricia Murtra, Lluís Armengol, Rubén D. Fernández-Montes, Mònica Gratacòs, Martí Pumarola, Roberto Cabrera, Rafael Maldonado, Josefa Sabrià, Xavier Estivill, Mara Dierssen

Overexpression of α3/α5/β4 nicotinic receptor subunits modifies impulsive-like behaviour

Xavier Viñals, Susanna Molas, Xavier Gallego, Rubén D. Fernández-Montes, Patricia Robledo, Mara Dierssen, Rafael Maldonado

(see below)

COMMUNICATIONS

Cyclic AMP levels reveal alterations of the central noradrenergic transmission in a mouse model of anxiety disorders

Molas, S. ; Gallego, X. ; Santos, M.; Dierssen, M.
Cajal Winter Conference, Benasque, 2008, Poster presentation

Nicotine involvement in anxiety-related behavior and drug dependence converge in the human CHRNA3/CHRNA5/CHRNB4 genetic cluster: evidence from transgenic mice

Molas, S. ; Gallego, X. ; Santos, M.; Dierssen, M.
VII Simposi de Neurobiologia de la Societat Catalana de Biología, Barcelona, 2008, Poster presentation

The human CHRNA3/CHRNA5/CHRNB4 genetic cluster is involved in the nicotine effects on neurocognitive processes: evidence from transgenic mice

Molas, S. ; Gallego, X. ; Santos, M.; Dierssen, M.
XIII Congreso de la Sociedad Española de Neurociencia (SENC), Tarragona, 2009, Poster presentation

The contribution of the human genomic cluster CHRNA3/CHRNA5/CHRNB4 to learning processes in a mouse model for nicotine addiction

Molas, S. ; Gallego, X. ; Santos, M.; Dierssen, M.
VII FENS FORUM, Amsterdam, 2010, Poster presentation

Gallego X, Molas S, Amador-Arjona A, Marks MJ, Robles N, Murtra P, et al. Overexpression of the CHRNA5/A3/B4 genomic cluster in mice increases the sensitivity to nicotine and modifies its reinforcing effects. Amino Acids. 2012 Aug;43(2):897-909.

Vinals X, Molas S, Gallego X, Fernandez-Montes RD, Robledo P, Dierssen M, et al. Overexpression of alpha3/alpha5/beta4 nicotinic receptor subunits modifies impulsive-like behavior. Drug Alcohol Depend. 2012 May 1;122 (3):247-252.