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

Xavier Viñals[1], Susanna Molas[2], Xavier Gallego[2], Rubén D. Fernández-Montes[2], Patricia Robledo [1,3], Mara Dierssen[2] and Rafael Maldonado[1]

[1]Laboratory of Neuropharmacology, Pompeu Fabra University (UPF), PRBB, Barcelona, Spain

[2]Genes and Disease Program, Center for Genomic Regulation (CRG-UPF), PRBB and CIBERER, Barcelona, Spain.

[3]Neuropsychopharmacology Research Program, IMIM (Hospital del Mar Research Institute), PRBB, Barcelona, Spain.

Corresponding Author:
Rafael Maldonado
Laboratori de Neurofarmacologia
Universitat Pompeu Fabra
PRBB
Calle Dr. Aiguader, 88
08003 Barcelona
SPAIN

Tel: + 34 93 316 08
FAX: + 34 93 316 0901
Email: rafael.maldonado@upf.edu

Abstract

Recent studies have revealed that sequence variants in genes encoding the α3/α5/β4 nicotinic acetylcholine receptor subunits are associated to nicotine dependence. In this study, we evaluated two specific aspects of executive functioning related to drug addiction (impulsivity and working memory) in transgenic mice over expressing α3/α5/β4 nicotinic receptor subunits. Impulsivity and working memory were evaluated in an operant delayed alternation task, where mice must inhibit responding between 2 and 8 sec in order to receive food reinforcement. Working memory was also evaluated in a spontaneous alternation task in an open field. Transgenic mice showed less impulsive-like behavior than wild-type controls, and this behavioral phenotype was related to the number of copies of the transgene. Thus, transgenic Line 22 (16-28 copies) showed a more pronounced phenotype than Line 30 (4-5 copies). Overexpression of these subunits in Line 22 reduced spontaneous alternation behavior suggesting deficits in working memory processing in this particular paradigm. These results reveal the involvement of α3/α5/β4 nicotinic receptor subunits in working memory and impulsivity, two behavioral traits directly related to the vulnerability to develop nicotine dependence.

Keywords: Nicotine dependence, gene variants, CHRNA3/A5/B4 gene cluster, nicotine acetylcholine receptor, delayed alternation.

1. Introduction

Drug addiction is recognized as a chronic disease characterized by major alterations in cognitive processing (Volkow et al., 2002; Robbins et al., 2008). Thus, increased attention to drug-paired stimuli and the memories of drug-related effects can induce craving and relapse even after long periods of abstinence (Everitt et al., 2007). Changes in other features of executive functioning such as inhibitory control and behavioral flexibility have also been reported to contribute to the cycle of addiction (Jentsch, and Taylor, 1999; Ersche et al., 2006; Dom et al., 2005).

Recent studies have revealed that sequence variants in genes of the CHRNA3/A5/B4 gene cluster, encoding the α3/α5/β4 human nicotinic acetylcholine receptor subunits are associated to nicotine dependence (Thorgeirsson et al., 2008; Thorgeirsson et al., 2010; Improgo et al., 2010) and lung cancer (Amos et al., 2008; Hung et al., 2008). However, it is not clear whether these gene variants promote nicotine dependence vulnerability, the main risk factor for lung cancer, or directly enhance lung cancer risk. The use of appropriate animal models could provide new insights to clarify the significance of this association.

In this study, we investigated whether the overexpression of α3/α5/β4 nicotinic acetylcholine receptor subunits would produce changes in two specific aspects of executive functioning, working memory and response inhibition that could be related to nicotine dependence vulnerability. For that purpose, we used an operant delayed alternation task in transgenic mice overexpressing these subunits and wild-type (WT) littermates, where animals are required to continuously change their response strategy in order to obtain food reinforcement (Weiss et al., 2005). Thus, mice must sustain attention on the previously emitted response (working memory) and inhibit premature responding during the delay requirement (response inhibition).

2. Materials and Methods

2.1 Animals

Male hybrid B6/SJL-F1J mice were housed in groups of 3-5 animals per cage under a reverse 12 h light/dark schedule (lights on 20:00 to 8:00) in controlled environmental conditions of humidity (50% - 70%) and temperature (21 ± 1ºC) with food and water supplied *ad libitum*. Transgenic mice were obtained in heterozygosity by standard pronucleus microinjection of the 111 kb BAC fragment inserted with the human cluster containing the three nicotinic receptor subunits (α3, α5, and β4) on B6/SJL-F1J genetic background, as previously described (Gallego et al., in press). 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). Two lines were generated carrying between 16-18 (Line 22 Tg) and 4-5 copies (Line 30 Tg) of the transgene, respectively, as detected by Slot-blot (data not shown). Similar mRNA overexpression of α3, α5 and β4 subunits was confirmed in both lines by RT-PCR. No significant differences between genotypes were found in endogenous α3, α5, and β4 subunits expression in the cerebral cortex when mouse Chrna3, Chrna5 and Chrnb4 were checked by qRT-PCR analysis. Western blot analysis confirmed increased expression of β4 and α3 subunits in specific regions of transgenic mice in both transgenic lines compared to WT mice (Gallego et al., in press). Binding assays using [^{3}H] nicotine showed an increase of two to three folds of nicotine binding sites in transgenic hippocampal membranes, as compared to WT with no differences in [^{3}H] MLA binding (Gallego et al 2011, in press).

The two transgenic lines (Line 22 and Line 30) were used in order to exclude positional effects. Hybrid founders were crossed using B6/SJL-F1J females and all experiments were performed using mice from the F2–F5 generation to attenuate littermate's genetic differences. The non-transgenic littermates obtained from crosses of males TgCHRNA3/A5/B4 mice and B6/SJL-F1J females served as controls. All experimental procedures were approved by the local ethical committee (CEEA-IMIM and CEEA-PRBB), and met the guidelines of the local (Catalan law 5/1995 and Decrees 214/97, 32/2007) and European regulations (EU directives 86/609 and 2001-486).

2.2 Western blot analysis of cytochrome P450 2A6 and α4, α6, α7, β2 and β3 nicotine acetylcholine receptor (nAChR) subunits in the prefrontal cortex

To determine if the changes in expression levels of the genes contained in the cluster could induce any compensatory modification in other nicotinic subtypes and subunits, the expression levels of the most relevant nicotinic subunits present in the central nervous system were evaluated in the prefrontal cortex tissue extracts from adult (3 month of age) TgCHRNA3/A5/B4 (Line 22; n = 5) and WT mice (n = 5). Cortical extracts were lysated in ice-cold RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS 0.1 M, pH 7.4). The samples were mixed in NuPAGE® LDS Sample Buffer (4x) and NuPAGE® Sample Reducing Agent (10x) (Invitrogen, Carlsbad, CA, USA). Equal total protein amounts (20 µg) were loaded per lane, separated on a NuPAGE® 4-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA) and transferred to an iBlot® Gel Transfer Stacks Nitrocellulose Regular membrane (Invitrogen, Israel). The nitrocellulose membranes were blocked (1 h, room temperature) with ODYSSEY® Blocking

Buffer (LI-COR Biosciences) and incubated at 4ºC overnight with the following rabbit antibodies: anti-nAChRα4 (1:1000, Abcam, Cambrige, UK), anti-nAChRα6 (1:10000, Millipore, Temeluca, CA, USA), anti-nAChRα7 (1:1000, Abcam, Cambridge, UK), anti-nAChRβ2 (1:800, AVIVA Systems, San Diego, CA, USA), anti-nAChRβ3 (1:10000, AVIVA Systems, San Diego, CA, USA), anti-cytochrome P450 2A6 (1:2000, Abcam, Cambridge, UK). Incubation for 1 h with the specific secondary IRDye® 680 donkey anti-rabbit (LI-COR Biosciences, Lincoln, NE, USA,) allowed detection using the ODYSSEY® Infrared Imaging System scanner. Ponceau S staining was used as loading control. Fluorescent bands were analyzed with the ImageJ image processing program.

2.3 Impulsive-like behavior

Mice were first food deprived to 85-90% of their free-feeding weight, and then trained in an operant delayed alternation paradigm during the dark phase of the light/dark cycle. The experiments were conducted in 6 mouse operant chambers (Med Associates Inc. Georgia, VT, USA), housed in sound-attenuated boxes equipped with a fan to provide ventilation and avoid ambient noise. The chambers were provided with a house-light, two nose poking holes (15 mm diameter) equidistantly placed on one of the walls, and a food magazine placed on the opposite wall, where 20 mg chocolate-flavored pellets (AIN-76A Rodent Tab Choc. Testdiet, Richmond, IN, USA) were delivered. The poking holes and the magazine were equipped with infrared photocells and lights, and responses were recorded by the computer using the MED-PC software. Accuracy (percent correct trials) and premature responding (latency to respond in sec) during the delay requirement were recorded. Mice were trained in daily sessions which

lasted until 50 reinforcers were delivered or after 30 min had elapsed, whichever occurred first. Animals were then returned to their home-cages and fed with standard chow (approximately 2-2.5 g each).

2.3.1 Magazine training

Shaping begun with mice receiving food pellets from the magazine every 20 sec during 3 sessions. Then they were trained to nose poke on either hole to obtain a food pellet on a continuous reinforcement schedule. During the third step, mice were reinforced for nose-poking only on the left or the right hole during 4 consecutive sessions, switching the active hole between sessions.

2.3.2 Alternation training

During this phase, mice learned to distribute responses between alternate locations. First, the choice opportunity (left or right hole) was signalled by illumination of the house-light. After the first response in one of the holes, the house-light was extinguished, the magazine-light was turned on, a food pellet was delivered, and a sound was presented during 1 sec. If the pellet was collected, the house light was illuminated after a 1 sec delay and the next trial begun. If mice nose-poked in either of the holes during the delay interval, or in the incorrect hole, the house-light was extinguished and the food cup light was illuminated, but no food was presented nor the sound activated. Mice were then required to turn away from the operands and nose-poke in the food magazine on the opposite wall to restart another trial. This procedure minimized the possibility that the animals' would use a lateralized position or other mediating responses as a strategy to alternate on the two holes. The criteria for acquisition of the alternation task was 1) a minimum of 40 reinforcers obtained per

session; 2) more than a 75% of correct responding 3) stability of responding during 2 consecutive sessions with less than 20% deviation from the mean.

2.3.3 Delayed alternation training

When the alternation criteria were met, four different delay intervals were introduced (2, 4, 6 and 8 sec) in a pseudorandom order during 15 days. Each delay was presented until a correct response was made, and the daily sessions were terminated when each delay was correctly completed for a total of 10 trials.

2.4 Locomotor activity

Locomotor activity was measured in an open-field consisting in a rectangular arena (70 cm long x 70 cm wide x 60 cm high) made of Plexiglas and covered with red plastic. The test was performed under low non-aversive lighting conditions (50 Lux). Adult TgCHRNA3/A5/B4 (Line 22; n = 12) and WT mice (n = 12) were monitored for 10 min and total distance travelled and time spent in each zone were registered using a video-tracking software (SMART, Panlab, SL., Spain).

2.5 Working memory

Taking into account that working memory can be measured as a concurrent processing task, we evaluated repeated explorations to two similar objects located in the center of an open field as an index of working memory in TgCHRNA3/A5/B4 (Line 22; n = 12) and WT mice (n = 12). We considered

exploration when a mouse directed the nose to the objects at a distance of no more than 2 cm. Sitting on the object was not considered as exploratory behavior. Each animal was placed on the centre of the field and during a 10 min session the sequence of object visits (Maccarrone et al., 2002) was recorded using a video-tracking software (SMART, Panlab, SL., Spain) and the object exploration was measured by the experimenter blind for the genotype of the animals. The arena and the objects were deeply cleaned between animals in order to avoid odor interferences. Consecutive visits to a previously explored object were thus recorded and plotted as frequency to revisit the same object either one, two, three or more consecutive times.

2.6 Statistical analysis

The data were analyzed using two-way repeated measures analysis of variance (ANOVA) with training day as a within-subjects factor and genotype as a between-subjects factor. Post-hoc comparisons between genotypes for each day were carried out using estimation of parameters. For the cumulative data (3-day blocks), one-way ANOVA was used to compare differences between genotypes. **ANOVA was also used to analyze locomotor activity data and to compare differences between genotypes in the working memory test.**

3. Results

3.1 Protein expression levels of cytochrome P450 2A6 and α4, α6, α7, β2 and β3 nAChR subunits

Previous studies from our laboratories (Gallego et al., in press) showed the overexpression of the α3, β4 and α5 nAChR subunits in different regions of the brain of TgCHRNA3/A5/B4 mice. Remarkably, the α3 nAChR subunit was overexpressed in the cerebral cortex of TgCHRNA3/A5/B4 (Line 22) mice. Because of a large body of evidences linking the prefrontal cortex with impulsivity (Reviewed in Perry et al., 2011), we have performed Western blot analysis to evaluate whether the protein expression levels of the nicotine-metabolizing enzyme involved in nicotine addiction (Thorgeirsson et al., 2010) cytochrome P450 2A6, and/or the α4, α6, α7, β2 and β3 nAChR subunits were also modified in the prefrontal cortex of TgCHRNA3/A5/B4 (Line 22) mice. As shown in Figure 1, no statistically significant changes were observed in the expression levels of these proteins.

3.2 Alternation training

All animals, irrespective of genotype or transgenic line, learned similarly the alternation task within 10 days of training. Indeed, the percent of correct trials (accuracy) increased as a function of training day from chance alternation (50% correct) on the first day, to about 80% correct on day 10. For both transgenic lines, two-way ANOVA revealed a significant effect of training day [Line 22: $F_{(9,252)} = 34.032$, $p<0.001$; Line 30: $F_{(9,261)} = 66.284$, $p<0.001$], but no significant effect of genotype [Line

22: $F_{(1,27)}$ = 1.517, NS ; Line 30: $F_{(1,29)}$ = 0.404, NS], nor interaction between factors [Line 22: $F_{(14,378)}$ = 1.082, NS ; Line 30:$F_{(9,261)}$ = 0.399, NS] were observed (Figure 2A and 3A).

3.3 Delayed alternation

Training on the delayed alternation task did not reveal significant differences between genotypes or transgenic lines with respect to errors due to alternation. Thus, accuracy was maintained from 80 to 90 % during the 15 days of training (data not shown). On the other hand, significant differences were observed with respect to premature responding when all delays were taken together. For Line 22, two-way ANOVA showed a significant main effect of training day [$F_{(14,336)}$ = 65.712; $p<0.001$] and genotype [$F_{(1,24)}$ = 7.012; $p<0.05$], without interaction between these two factors [$F_{(14,336)}$ = 1.142; NS]. Post-hoc analysis revealed a significant decrease in the number of premature responses in Line 22 mice in comparison with WT on day 2 ($p<0.001$), 4, 5, 6, 11, 14 and 15 ($p<0.05$) (Figure 2B). One-way ANOVA for cumulative data on premature responding in blocks of 3 consecutive days (inset) showed significant differences between genotypes on days 4-6 ($p<0.05$), 7-9 ($p<0.05$), 10-12 ($p<0.05$) and 13-15 ($p<0.05$).

In contrast, two-way ANOVA showed for Line 30 a significant main effect of training day [$F_{(14,378)}$ = 95.150; $p<0.001$], without effect of genotype [$F_{(1,27)}$ = 1.517; NS] nor interaction between these factors [$F_{(14,378)}$ = 1.082; NS] (Figure 3B). Post-hoc analysis just revealed a decrease in premature responding on days 7 and 10 ($p < 0.05$). One-way ANOVA for cumulative data on premature responding in blocks of 3

consecutive days (inset) showed no significant differences between genotypes on any block.

3.4 Locomotor activity

Since differences in the delayed alternation test in Line 22 may be influenced by changes in locomotor activity, we analyzed this parameter in an open-field paradigm. One-way ANOVA revealed no differences regarding total travelled distance between WT and Line 22 transgenic mice [$F_{(1,23)}$ = 1.604; NS, one-way ANOVA] (Figure 4A). Moreover, the percentage of time spent in periphery and centre were not different between genotypes [$F_{(1,23)}$ = 0.504; NS, one-way ANOVA] (Figure 4B), thus indicating no changes in locomotor activity or anxiety-like behavior in transgenic mice overexpressing the human *CHRNA3/A5/B4* genomic cluster.

3.5 Working memory

To address working memory we analyzed the frequency of revisits to the same object for one, two, three or more than 3 consecutive times. A one-way ANOVA did not reveal any difference between WT and transgenic animals from Line 22 when evaluating a low number of revisits to the same object (one revisit $F_{(1,23)}$ = 0.103; NS; two revisits $F_{(1,23)}$ = 2.024; NS and three revisits $F_{(1,23)}$ = 0.021; NS). However, a significant difference between WT and Line 22 transgenic mice was revealed when analyzing number of revisits superior to 3 [$F_{(1,23)}$ = 6.778; $p < 0.05$] (Figure 5).

4. Discussion

In this study, we reveal specific changes on response inhibition in transgenic mice overexpressing α3/α5/β4 nicotinic acetylcholine receptor subunits using an operant delayed alternation task (Weiss et al., 2005). In this paradigm, mice are required to continuously change their response strategy in order to obtain food reinforcement. They must sustain attention on the previously emitted response and inhibit premature responding during the delay requirement. Premature responding during the delay task is associated with disruptions in the control of response inhibition and provides a measure of impulsivity (Evenden, 1999; Robbins, 2002; Lambourne et al., 2007). Transgenic mice decreased premature responses in this paradigm, which suggests an improvement in the control of impulsivity. Interestingly, the improvement of this behavioral response was directly related to the number of copies of the transgene. Thus, Line 22 carrying between 16-18 copies of the transgene showed a reliable decrease in the number of premature responses during most of the training period, whereas a modest improvement of the performance was revealed in Line 30, which carries only 4-5 copies of the transgene. **The fact that Line 22 transgenic mice have a more pronounced behavioral phenotype compared to Line 30 transgenic mice may also be attributable to changes in the functionality of the subunits rather than simply to their protein expression levels since the seizure response to high doses of nicotine is increased in L22 transgenic mice compared to L30 (data not shown).**

Recent studies have revealed a direct relationship between impulsivity and the vulnerability to develop drug addiction (Belin et al., 2008). The decreased impulsivity of these transgenic mice reveals the specific involvement of α3/α5/β4 nicotinic acetylcholine receptor subunits in this personality trait directly related to drug addiction vulnerability. **Furthermore, studies in animal models of nicotine addiction have**

implicated the α5 subunit in the medial habenula (mHb) and its projection area, the interpeduncular nucleus, in the reinforcing properties of nicotine (Fowler et al., 2011). Thus, knockout mice lacking the α5 subunit showed increased nicotine self-administration at higher doses with respect to WT mice, and this effect was reversed by the selective re-expression of this subunit in the mHb of knockout mice. In addition, rats with a selective knockdown of the α5 subunit in this structure also exhibited a marked increase in nicotine consumption as compared to control rats (Fowler et al., 2011). These data provide strong evidence for an inhibitory role of the a5 subunit in the medial habenula-interpeduncular circuit in nicotine intake.

A role for the mHb in impulsive behavior has also been recently put forward. Thus, intra-habenula neonatal administration of ibotenic acid or nicotine produces a selective lesion of this structure, and leads to increased impulsivity (Lee and Goto, 2011). However, our transgenic models do not show changes in the expression of α3/α5/β4 subunits in this region with respect to WT mice (Gallego et al, in press), thus discarding this mechanism in the observed phenotype.

The operant delayed alternation task used in this study allows the evaluation of working memory related to medial prefrontal cortex functionality (Granon et al., 1994; Goldman-Rakic, 1995). Thus, the similar accuracy observed in transgenic mice and WT littermates during the delay training period reveals that the overexpression of α3/α5/β4 nicotinic acetylcholine receptor subunits does not produce alterations in this **particular type of working memory. In order to further characterize this behavioral trait, we analyzed spontaneous alternation in the transgenic line showing the most relevant phenotype (Line 22) and found significant differences in these transgenic mice when a high number of revisits were analyzed. The discrepant results obtained in**

these two paradigms may be attributed to differences in the motivational component of both tasks. Thus, in the delayed alternation task, mice must alternate their responses on two levers in order to obtain a reward, while in the working memory task mice are not rewarded for correct alternation.

Finally, our locomotor activity data showing that total distance travelled in the open field was similar between genotypes suggest that the decrease in premature responding observed in transgenic mice in the delayed alternation task cannot be attributed to possible differences in locomotion. Importantly, from the molecular point of view there were not significant changes in expression of other nicotinic receptor subunits in the prefrontal cortex, further suggesting that α3/α5/β4 nicotinic receptor subunits are related to the observed phenotype.

In summary, our study reveals the involvement of α3/α5/β4 nicotinic receptor subunits in impulsivity, a behavioral trait directly related to the vulnerability to develop nicotine dependence. This new behavioral role identified for the α3/α5/β4 subunits provides a functional link for the association of genes variants of these subunits and nicotine dependence (Thorgeirsson et al., 2008). Therefore, these genomic variants could promote the development of nicotine dependence.

5. Acknowledgments

This work was supported by the DG Research of the European Commission (PHECOMP, no. LHSM-CT-2007-037669), the Spanish 'Instituto de Salud Carlos III' (RD06/001/001, PI082038 and PI10/01708), the Spanish 'Ministerio de Educación y Ciencia' (no. SAF2007-64062, SAF2010-16427), the Catalan Government (SGR2009-00131, SGR2009-1313) and the ICREA Foundation (ICREA Academia-2008).

6. References

Amos, C.I., Wu, X., Broderick, P., Gorlov, I.P., Gu, J., Elisen, T., Dong, Q., Zhan, Q., Gu, X., Vijayakrishnan, J., Sullivan, K., Matakidou, A., Wang, Y., Mills, G., Doheny, K., Tsai, Y.Y., Chen, W.V., Shete, S., Siptz, M.R., Houlston R.S., 2008. Genome-wide association scan of tag SNPs identifies a susceptibility locus for lung cancer at 15q25.1. Nat. Genet. 40, 616-622.

Belin, D., Mar, A.C., Dalley, J.W., Robbins, T.W., Everitt, B.J., 2008. High impulsivity predicts the switch to compulsive cocaine-taking. Science 320, 1352-1355.

Dom, G., Sabbe, B., Hulstijn, W., van den Brink, W., 2005. Substance use disorders and the orbitofrontal cortex: systematic review of behavioral decision-making and neuroimaging studies. Br J Psychiatry 187, 209-20.

Ersche, K.D., Clark, L., London, M., Robbins, T.W., Sahakian, B.J., 2006. Profile of executive and memory function associated with amphetamine and opiate dependence. Neuropsychopharmacology 31, 1036–1047.

Evenden, J., 1999. Impulsivity: a discussion of clinical and experimental findings. J. Psychopharmacol. 13, 180–192.

Everitt, B.J., Hutcheson, D.M., Ersche, K.D., Pelloux, Y., Dalley, J.W., Robbins, T.W., 2007. The orbital prefrontal cortex and drug addiction in laboratory animals and humans. Ann. N Y Acad Sci. 1121, 576-97.

Fowler, C.D., Lu, Q., Johnson, P.M., Marks, M.J., Kenny, P.J., 2011. Habenular α5 nicotinic receptor subunit signalling controls nicotine intake. Nature 471, 597-601.

Gallego, X., Molas, S., Amador-Arjona, A., Robles, N., Murtra, P., Armengol, L., Fernández-Montes, R., Gratacòs, M., Pumarola, M., Cabrera, R., Maldonado, R., Sabrià, J., Estivill, X., Dierssen, M. Overexpression of the CHRNA3/A5/B4 genomic cluster increases nicotine self-administration in mice. PLoS ONE (in press).

Goldman-Rakic, P.S., 1995. Cellular basis of working memory. Neuron 14, 477– 485.

Granon, S., Vidal, C., Thinus-Blanc, C., Changeux, J-P., Poucet, B., 1994. Working memory, response selection and effortful processing in rats with medial prefrontal lesions. Behav. Neurosci. 5, 883– 891.

Hung, R.J., McKay, J.D., Gaborieau, V., Boffetta, P., Hashibe, M., Zaridze, D., Mukeria, A., Szeszenia-Dabrowska, N., Lissowska, J., Rudnai, P., Fabianova, E., Mates, D., Bencko, V., Foretova, L., Janout, V., Chen, C., Goodman, G., Field, J.K., Liloglou, T., Xinarianos, G., Cassidy, A., McLaughlin, J., Liu, G., Narod, S., Krokan, H.E., Skorpen, F., Elvestad, M.B., Hveem, K., Vatten, L., Linseisen, J., Clavel-Chapelon, F., Vineis, P., Bueno-de-Mesquita, H.B., Lund, E., Martinez, C., Bingham, S., Rasmuson, T., Hainaut, P., Riboli, E., Ahrens, W., Benhamou, S., Lagiou, P., Trichopoulos, D., Holcátová, I., Merletti, F., Kjaerheim, K., Agudo, A., Macfarlane, G., Talamini, R., Simonato, L., Lowry, R., Conway, D.I., Znaor, A., Healy, C., Zelenika, D., Boland, A., Delepine, M., Foglio, M., Lechner, D., Matsuda, F., Blanche, H., Gut, I., Heath, S., Lathrop, M., Brennan, P., 2008. A susceptibility locus for lung cancer maps to nicotinic acetylcholine receptor subunit genes on 15q25. Nature 452, 633-637.

Improgo M.R., Scofield M.D., Tapper A.R., Gardner P.D., 2010. The nicotinic acetylcholine receptor CHRNA5/A3/B4 gene cluster: dual role in nicotine addiction and lung cancer. Prog. Neurobiol. 92, 212-226.

Jentsch, J.D., Taylor, J.R., 1999. Impulsivity resulting from frontostriatal dysfunction in drug abuse: Implications for the control of behavior by reward-related stimuli. Psychopharmacology (Berl.) 146, 373–390.

Lee, Y.A., Goto, Y., 2011. Neurodevelopmental disruption of cortico-striatal function caused by degeneration of habenula neurons. PLoS One, 6, e19450.

Perry, J.L., Joseph, J.E., Jiang, Y., Zimmerman, R.S, Kelly, T.H., Darna, M., Huettl, P., Dwoskin, L.P., Bardo, M.T., 2011. Prefrontal cortex and drug abuse vulnerability: translation to prevention and treatment interventions. Brain Res. Rev. 65, 124-49.

Robbins, T.W., 2002. The 5-choice serial reaction time task: behavioral pharmacology and functional neurochemistry. Psychopharmacology (Berl.) 163, 362–380.

Robbins, T.W., Ersche, K.D., Everitt, B.J., 2008. Drug addiction and the memory systems of the brain. Ann. NY Acad. Sci. 1141, 1-21.

Thorgeirsson, T.E., Geller, F., Sulem, P., Rafnar, T., Wiste, A., Magnusson, K.P., Manolescu, A., Thorleifsson, G., Stefansson, H., Ingason, A., Stacey, S.N., Bergthorsson, J.T., Thorlacius, S., Gudmundsson, J., Jonsson, T., Jakobsdottir, M.,

Saemundsdottir, J., Olafsdottir, O., Gudmundsson, L.J., Bjornsdottir, G., Kristjansson, K., Skuladottir, H., Isaksson, H.J., Gudbjartsson, T., Jones, G.T., Mueller, T., Gottsäter, A., Flex, A., Aben, K.K., de Vegt, F., Mulders, P.F., Isla, D., Vidal, M.J., Asin, L., Saez, B., Murillo, L., Blondal, T., Kolbeinsson, H., Stefansson, J.G., Hansdottir, I., Runarsdottir, V., Pola, R., Lindblad, B., van Rij, A.M., Dieplinger, B., Haltmayer, M., Mayordomo, J.I., Kiemeney, L.A., Matthiasson, S.E., Oskarsson, H., Tyrfingsson, T., Gudbjartsson, D.F., Gulcher, J.R., Jonsson, S., Thorsteinsdottir, U., Kong, A., Stefansson, K., 2008. A variant associated with nicotine dependence, lung cancer and peripheral arterial disease. Nature 452, 638-642.

Thorgeirsson, T.E., Gudbjartsson, D.F., Surakka, I., Vink, J.M., Amin, N., Geller, F., Sulem, P., Rafnar, T., Esko, T., Walter, S., Gieger, C., Rawal, R., Mangino, M., Prokopenko, I., Mägi, R., Keskitalo, K., Gudjonsdottir, I.H., Gretarsdottir, S., Stefansson, H., Thompson, J.R., Aulchenko, Y.S., Nelis, M., Aben, K.K., den Heijer, M., Dirksen, A., Ashraf, H., Soranzo, N., Valdes, A.M., Steves, C., Uitterlinden, A.G., Hofman, A., Tönjes, A., Kovacs, P., Hottenga, J.J., Willemsen, G., Vogelzangs, N., Döring, A., Dahmen, N., Nitz, B., Pergadia, M.L., Saez, B., De Diego, V., Lezcano, V., Garcia-Prats, M.D., Ripatti, S., Perola, M., Kettunen, J., Hartikainen, A.L., Pouta, A., Laitinen, J., Isohanni, M., Huei-Yi, S., Allen, M., Krestyaninova, M., Hall, A.S., Jones, G.T., van Rij, A.M., Mueller, T., Dieplinger, B., Haltmayer, M., Jonsson, S., Matthiasson, S.E., Oskarsson, H., Tyrfingsson, T., Kiemeney, L.A., Mayordomo, J.I., Lindholt, J.S., Pedersen, J.H., Franklin, W.A., Wolf, H., Montgomery, G.W., Heath, A.C., Martin, N.G., Madden, P.A., Giegling, I., Rujescu, D., Järvelin, M.R., Salomaa, V., Stumvoll, M., Spector, T.D., Wichmann, H.E., Metspalu, A., Samani, N.J., Penninx, B.W., Oostra, B.A., Boomsma, D.I., Tiemeier, H., van Duijn, C.M., Kaprio, J., Gulcher,

J.R.; ENGAGE Consortium, McCarthy, M.I., Peltonen, L., Thorsteinsdottir, U., Stefansson, K., 2010. Sequence variants at CHRNB3-CHRNA6 and CYP2A6 affect smoking behavior. Nat. Genet. 42, 448-53

Volkow, N.D., Fowler, J.S., Wang, G.J., Goldstein, R.Z., 2002. Role of dopamine, the frontal cortex and memory circuits in drug addiction: insight from imaging studies. Neurobiol. Learn. Mem. 78, 610-24.

Weiss, B., Stern, S., Cox, C., Balys, M., 2005. Perinatal and lifetime exposure to methylmercury in the mouse: behavioral effects. Neurotoxicology 26, 675–690.

7. Figure Legends

Figure 1. Western blot analysis of cytochrome P450 2A6 and α4, α6, α7, β2 and β3 nAChR subunits protein expression in the prefrontal cortex in TgCHRNA3, Line 22. (A) Bar graphs representing the densitometric analysis (arbitrary units) of Western blots of wild-type (WT; white bars) and TgCHRNA3/A5/B4 (TG; striped bars) mice. Data represent mean ± SEM *p < 0.05 one-way ANOVA between genotypes. (B) Representative Western blots of the analyzed proteins and Ponceau S staining of the membrane.

Figure 2. Performance on the operant delayed alternation task in Line 22 wild-type (WT) and Line 22 transgenic mice (TG). (A) Accuracy in alternation training without delays during 10 days in Line 22 WT (n = 15) and Line 22 TG (n = 15) mice. (B) Premature responding during 15 days considering all delays together (2, 4, 6 and 8 sec) in Line 22 WT (n = 13) and Line 22 TG (n = 13) mice. The inset shows premature responding in blocks of 3 days during the 15 days. The data represent mean ± SEM. *p<0.05; *** p<0.001 vs. Line 22 WT (estimation of parameters).

Figure 3. Performance on the operant delayed alternation task in Line 30 wild-type (WT) and Line 30 transgenic mice (TG). (A) Accuracy in alternation training without delays during 10 days in Line 30 WT (n = 15) and Line 30 TG (n = 16) mice. (B) Premature responding during 15 days considering all delays together (2, 4, 6 and 8 sec) in Line 30 WT (n = 15) and Line 30 TG (n = 14) mice. The inset shows premature responding in blocks of 3 days during the 15 days. The data represent mean ± SEM.

Figure 4. Locomotor activity and anxiety behavior were evaluated on the open field test in wild-type (WT) and Line 22 transgenic mice (TG). (A) Total distance travelled and (B) percentage of time spent in the periphery and centre was calculated over a 10 minutes session in Line 22 (n = 12 mice per genotype). The data represent mean ± SEM.

Figure 5. Spontaneous alternation on two similar objects located on the centre of an open field arena. The data represent mean ± SEM of the frequency of revisits to the same object during a 10 minutes session in wild-type (WT; n = 12) and Line 22 transgenic mice (TG; (n = 12). *** p < 0.001 vs. WT.