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Genetic variation within the *Chrna7* gene modulates nicotine reward-like phenotypes in mice[1]

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Mortality from tobacco smoking remains the leading cause of preventable death in the world, yet current cessation therapies are only modestly successful, suggesting new molecular targets are needed. Genetic analysis of gene expression and behavior identified *Chrna7* as potentially modulating nicotine place conditioning in the BXD panel of inbred mice. We used gene targeting and pharmacological tools to confirm the role of *Chrna7* in nicotine conditioned place preference (CPP). To identify molecular events downstream of *Chrna7* that may modulate nicotine preference, we performed microarray analysis of α7 knock-out (KO) and wild-type (WT) nucleus accumbens (NAc) tissue, followed by confirmation with quantitative polymerase chain reaction (PCR) and immunoblotting. In the BXD panel, we found a putative *cis* expression quantitative trait loci (eQTL) for *Chrna7* in NAc that correlated inversely to nicotine CPP. We observed that gain-of-function α7 mice did not display nicotine preference at any dose tested, whereas conversely, α7 KO mice demonstrated nicotine place preference at a dose below that routinely required to produce preference. In B6 mice, the α7 nicotinic acetylcholine receptor (nAChR)-selective agonist, PHA-543613, dose-dependently blocked nicotine CPP, which was restored using the α7 nAChR-selective antagonist, methyllycaconitine citrate (MLA). Our genomic studies implicated a messenger RNA (mRNA) co-expression network regulated by *Chrna7* in NAc. Mice lacking *Chrna7* demonstrate increased insulin signaling in the NAc, which may modulate nicotine place preference. Our studies provide novel targets for future work on development of more effective therapeutic approaches to counteract the rewarding properties of nicotine for smoking cessation.

Keywords: α7 Nicotinic acetylcholine receptor, behavioral genetics, BXD panel, *Chrna7*, conditioned place preference, genomics, insulin, nicotine, quantitative trait loci, reward

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Nicotine dependence as a result of tobacco use is the leading cause of preventable death in the world, contributing to 90% of lung cancer cases in the United States and 49 400 deaths from secondhand smoke per year (National Institutes of Health 2012). Several studies have demonstrated that genetic influences on nicotine addiction and dependence exist, with trait heritabilities between 46% and 84% (Heath & Martin 1993; Kendler *et al.* 1999; Swan *et al.* 1997; True *et al.* 1999). Although roughly 70% of adult smokers aspire to quit smoking, only 4–7% are successful without medication and only 25% of those using current cessation therapies are able to abstain from smoking for 6 months (American Cancer Society 2012). The inability to quit smoking is thought to be because of high rates of relapse from difficulty in managing cravings and withdrawal symptoms (National Institutes of Health 2008); thus, more effective treatments are required.

It has been hypothesized that associative cues in smokers can maintain drug-seeking behavior and reinforcement, even in the absence of nicotine (Caggiula *et al.* 2001; Rose & Corrigall 1997). A conditioned stimulus choice test, conditioned place preference (CPP), has been used in animals since the early 1940s to model appetitive reward-like properties of drugs of abuse (Rossi & Reid 1976; Spragg 1940). Thus, using CPP to test an animal's drug-free response to contextual cues associated with nicotine may lead to discovery of neural pathways that play a role in nicotine reward and reinforcement (Bardo & Bevins 2000).

Behavioral genetics studies in animal models have been widely used to study the role of specific nicotinic subunits or other genes in nicotine's reward-like properties or other behaviors (Changeux 2010). Additionally, several human studies have associated the genetic variation within multiple nicotinic acetylcholine receptor (nAChR) genes with different nicotine dependence phenotypes (Amos *et al.* 2008; Berrettini *et al.* 2008; Bierut *et al.* 2007; Ehringer *et al.* 2007; Hung *et al.* 2008; Saccone *et al.* 2007; Saccone *et al.* 2010; Zeiger *et al.* 2008). However, few forward genetics approaches have been used to identify novel targets for intervention in nicotine behaviors. Modern genetic panels, together with high-density genotyping and use of expression

genetics, have improved the prospects of using forward genetics to identify gene networks modulating complex traits such as nicotine dependence (Broide *et al.* 2002; Carlborg *et al.* 2005; Hitzemann *et al.* 2004).

To identify gene networks involved in nicotine dependence, we used a combination of behavioral genetics and pharmacological studies in mice, together with genetic analysis of gene expression. Our results implicate genetic variation in *Chrna7* messenger RNA (mRNA) expression and its potential regulation of insulin signaling as modulators of nicotine CPP. These studies may have important implications for understanding and treating nicotine dependence in humans.

Materials and methods

Mice

For all studies, male mice were housed three to five per cage and allowed at least a 1-week acclimation period to the vivarium following shipment to Virginia Commonwealth University (VCU). Mice were maintained on a 12-h light/dark cycle with *ad libitum* access to food and water. Adult mice were tested or had tissues harvested between 7 and 12 weeks of age during their light phase. C57BL/6J (B6, Stock No. 000664), DBA/2J (D2, Stock No. 000671) and BXD (B6 × D2) recombinant inbred mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). *Chrna7* knock-in, gain-of-function (α7 KI) mice (*Chrna7* L250T +/−), bred from heterozygous breeding pairs, were obtained from Baylor College of Medicine (Houston, TX, USA) (Broide *et al.* 2002). *Chrna7* homozygous knock-out (α7 KO) mice (B6.129S7-Chrna7tm1Bay/J, Stock No. 003232) were obtained from Jackson Laboratories; heterozygote breeding pairs were obtained from wild-type (WT) and KO mice that were bred and genotyped at VCU. Both α7 KI and α7 KO mice were backcrossed to the background strain, C57BL/6J, for an additional 8–10 generations and wild-type littermates (α7 WT) were used as controls. The animal facility was approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Experiments were performed during the light cycle and approved by the Institutional Animal Care and Use Committee of VCU.

Drugs and chemicals

(−)-Nicotine hydrogen tartrate salt and methyllycaconitine citrate (MLA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). PHA-543613 [*N*-[(3R)-1-azabicyclo[2.2.2]oct-3-yl]furo[2,3-c]pyridine-5-carboxamide] and cocaine hydrochloride were obtained from the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD, USA). All drugs were dissolved in a vehicle of physiological saline (0.9% sodium chloride), filter sterilized and administered at a volume of 0.1 ml per 10 g of mouse mass. Nicotine, PHA-543613 and MLA were administered subcutaneously (s.c.), whereas cocaine was given intraperitoneally (i.p.). All doses are expressed as the free base of the drug.

Place conditioning experiments

For all place conditioning experiments with BXD strains, α7 KO, KI and WT mice, a 5-day paradigm was performed as described previously (Kota *et al.* 2007). Each animal received cage enrichment, and on Wednesday, Thursday and Friday of the week prior to place conditioning testing, the experimenter handled each mouse for approximately 2 min. The experimental apparatus (Med-Associates, St. Albans, VT, USA, ENV3013) consisted of white and black chambers (20 × 20 × 20 cm each), which differed in floor texture (white mesh and black rod). The chambers were separated by a smaller gray chamber with a smooth PVC floor and partitions that allowed access to the black and white chambers. Briefly, on day 1 (preconditioning day), mice were placed in the center chamber for 5 min, partitions were lifted and mice were allowed to roam freely for 15 min. The time spent in the white and black chambers was used to establish baseline chamber preferences, if any. Mice were separated into vehicle and drug groups such that initial chamber biases in each group were approximately balanced. On days 2–4 (conditioning days), twice per day, mice were injected with vehicle or drug and subsequently paired with either the white or black chamber, where they were allowed to roam for 15 min. Vehicle-treated animals were paired with saline in both chambers and drug-treated animals received saline in one chamber and nicotine in the opposite chamber. Pairing of the drug with either the black or white chamber was randomized within the drug-treated group of mice. On day 5 (test day), mice did not receive an injection. They were placed into the center chamber for 5 min, the partitions were lifted and they were allowed to roam freely for 15 min. Time spent in each chamber was recorded. Additional experimental details are provided in Appendix S1.

Several studies evaluating the role of α7 nAChRs in nicotine CPP were conducted. We tested nicotine CPP (0.1 and 0.5 mg/kg) in α7 KO, KI and WT mice using the procedure described above. In separate studies, C57BL/6J mice were pretreated with either saline or PHA-543613 (4.0, 8.0 and 12.0 mg/kg) 15 min before nicotine administration (0.1 or 0.5 mg/kg). Finally, C57BL/6J mice were pretreated with either saline or MLA (10 mg/kg), 15 min later, treated with PHA-543613 (12 mg/kg), and another 15 min later, treated with nicotine (0.5 mg/kg).

A similar procedure was used for cocaine CPP as a positive control. We tested cocaine CPP (10 mg/kg) in α7 KO, KI and WT mice. Finally, C57BL/6J mice were pretreated with either saline or PHA-543613 (12 mg/kg), and 15 min later, received cocaine (10 mg/kg). Doses were chosen based on those previously produced reliable CPP for nicotine (Grabus *et al.* 2006; Walters *et al.* 2006) and cocaine (Sora *et al.* 2001).

Behavioral data analysis and statistics

For each drug-treated mouse, preference scores were calculated as time spent in the drug-paired side on test day minus time spent in the drug-paired side during baseline. For each saline-treated mouse, preference scores were calculated as the average of the white side on test day minus the white side during baseline and the black side on test day minus the black side during baseline. Strain means were then calculated. As within-strain variability for place conditioning across the BXD panel was high for both nicotine and saline treatments, we performed a subtraction of BXD strain means; nicotine preference scores minus saline preference scores. This was performed to normalize as well as remove possible confounds of the saline phenotype on the nicotine phenotypic measures. With one exception, none of the drugs used herein caused significant alterations in locomotor activity on test day at the doses tested (*t*-tests or one-way ANOVA, where appropriate, with treatment as the factor; see Table S1), Therefore, time spent in either chamber was not confounded by locomotor activity. BXD strains 27, 32 and 8 did demonstrate statistical differences by treatment (saline vs. nicotine) in locomotor activity on test day; however, these differences did not impact heritability calculations (Table S2), or quantitative trait loci (QTL) mapping results (data not demonstrated); thus, we could not justify removing these strains from our dataset. Statistical analysis of all behavioral studies was performed using one- or two-way Eanalysis of variance (ANOVA), where appropriate. If a one-way ANOVA was significant, an appropriate *post hoc* test was performed (Dunnett's *post hoc* test for comparisons vs. control and Tukey's HSD for between-group comparisons when more than two groups existed). *Post hoc P* values of <0.05 were considered to be statistically significant.

QTL mapping, correlations and heritability calculations

In order to identify putative genes underlying nicotine place conditioning, QTL mapping and genetic correlations were performed using BXD strain means for behaviors and expression data using GeneNetwork (Chesler *et al.* 2004) and R/qtl (Arends *et al.* 2010). Heritabilities for nicotine and saline place conditioning phenotypes were estimated using the intraclass correlation coefficient method

at $\alpha = 0.05$ (Lynch & Walsh 1998; Smith 1957) using the intraclass correlation coefficients (ICC) package in R (Wolak 2012).

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR), microarrays, data analysis and network generation

For qRT-PCR experiments, single nucleus accumbens (NAc) samples from 9-week old, untreated C57BL/6J, DBA/2J, α7 KO and WT mice were microdissected on ice and immediately flash-frozen in liquid nitrogen using a protocol described previously (Kerns *et al.* 2005). Samples were homogenized with a Polytron® (Kinematica AG, Bohemia, NY, USA) and extracted using a guanidine/phenol/chloroform method (STAT-60, Tel-Test, Inc., Friendswood, TX, USA). Each RNA liquid layer was added to an RNeasy Mini Column (Qiagen) for cleanup and elution of total RNA. RNA quality and purity was determined using an Experion Automated Electrophoresis Station (Bio-Rad, Hercules, CA, USA) and a Nanodrop 2000 (Thermo Scientific, Rockville, MD, USA). A total of 1 μg of RNA was converted to complementary DNA (cDNA) using the iScript cDNA synthesis kit containing random hexamers (Bio-Rad) according to manufacturer's instructions. Primer sequences, Tm's, amplicon sizes and cDNA dilutions used for each gene are listed in Table S10. Primer efficiencies were between 90% and 110% and each primer set resulted in only one PCR product. Data analysis was performed using the $2^{-[\Delta\Delta CT]}$ method (Heid *et al.* 1996). Statistical analysis of qRT-PCR data was performed using a Student's *t*-test between the two strains tested.

Affymetrix Mouse 430A 2.0 microarrays were performed on α7 KO and WT single NAc samples from individual animals ($n = 5$/genotype). Sample collection and RNA extraction were performed as described above. All RNA RQI values were >9.0, 260/280 ratios were between 1.9 and 2.1, and 260/230 ratios were >2.0. Samples were randomized at all possible steps and 100 ng of RNA input were used for each 16-h in vitro transcription reaction. Remaining steps were performed according to the manufacturer's protocol. All microarrays passed each quality control measure and Pearson correlations of robust multi-array average signals between single chips were ≥0.996. Pairwise significance-scores (s-scores) between KO and WT chips were generated using the s-score package in R, Version 2.14.2 (Kerns *et al.* 2003). A one-class statistical analysis of microarrays against a mean = 0 and 100 permutations, was performed in the MultiExperiment Viewer (MeV, Boston, MA, USA). A delta of 0.314, a false-discovery rate of 9.8% and s-scores ≥ |1.5| were used to identify significantly differentially regulated genes (Table S9). Gene network construction was performed using Ingenuity Pathway Analysis (Ingenuity, Redwood City, CA, USA), which uses Fisher's exact test to determine the probability of input genes belonging to the network by chance (Ingenuity Pathway Analysis 2005).

Immunoblotting

Nucleus accumbens from single untreated α7 KO or WT mice were microdissected and flash-frozen in liquid nitrogen as described above. Samples were triturated in 100 ul of cold 1× LDS (Life Technologies, Grand Island, NY, USA) containing 2× Halt protease and phosphatase inhibitor cocktail and 10 mM ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific). Each sample was passed through a 28 g syringe until brain tissue was no longer visible upon quick-spin centrifugation. Protein concentrations of whole sample homogenates were determined using the bicinchoninic acid assay (Thermo Fisher Scientific). Samples were balanced with 1× LDS, reduced with 50 mM dithiothreitol and boiled for 10 min. For each antibody used herein, it was determined that 20 μg of protein lie within the linear range of detection, thus 20 μg of protein were loaded per lane on a 4%–12% NuPage bis–tris gel (Life Technologies). Using 1× MOPS running buffer, electrophoresis was performed at 150 V. The gel was transferred to a PVDF membrane at 10 V for 24 h using a freshly prepared transfer buffer containing 10% methanol. Coomassie staining of the gel and Ponceau staining of the membrane indicated efficient and even transfer. Prior to each primary antibody incubation, the membrane was blocked with 5% BSA in 1× wash buffer (TBS-T containing 0.3% Tween 20 and 1.5 M NaCl) for 1 h at room temperature. Primary and secondary antibody catalog numbers, dilutions and incubation times are provided in Table S11. Immunoblots were imaged on Kodak film using the chemiluminescent enhanced chemiluminescence (ECL) prime reagent (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and quantified using ImageJ processing and analysis software, Version 1.440 (National Institutes of Health, Bethesda, MD, USA). All proteins were normalized to the loading control, β-actin (ACTB). Statistical analysis of immunoblot data was performed using a Student's *t*-test between the two strains tested.

Results

Behavioral genetic analysis of nicotine place conditioning in BXD mice

Following conditioning with either saline–saline or saline–nicotine and subsequent testing in the absence of drug, nicotine place preference scores (Fig. 1a) were calculated across BXD strains tested ($n = 23$ BXD strains, $n = 6$–12 per treatment). Strains with positive scores are interpreted as having a preference for nicotine or saline, while negative scores indicate a place aversion. These scores followed a continuous distribution, indicative of a polygenic trait, and the nicotine scores were quite distinct from results seen with saline. The progenitor strains, C57BL/6J and DBA/2J, demonstrated divergent phenotypes. Some BXD strains demonstrated a trend for mild preference to the saline-treated side. The nicotine–saline subtracted phenotype (Fig. 1b) in fact represents the nicotine response as the unsubtracted nicotine scores and subtracted scores were tightly genetically correlated (Pearson's $r = 0.920$, $P = 9.84\text{E}{-14}$). The mean heritability for nicotine place preference was 18.7% (Table S2).

Genetic correlation analysis of nicotine place conditioning with traits within the GeneNetwork phenotype database (www.GeneNetwork.org) revealed that nicotine place preference significantly correlates inversely to the number of cholinergic neurons in multiple dorsal and ventral striatal sections (Table 1), suggesting a link between the nicotine place preference and cholinergic signaling in the striatum. However, nicotine preference scores also significantly correlated positively to a number of other phenotypes including exploratory behaviors (Table S3), suggesting a complex genetic architecture of the phenotype. Furthermore, no significant behavioral QTL was identified for the nicotine place conditioning phenotype (maximum LRS of 10.4 at 18.8 Mb on Chr X).

Basal Chrna7 mRNA expression in the NAc is inversely correlated with nicotine place conditioning in BXD mice

We previously generated basal genomic expression profiles across the mesolimbocortical dopamine (DA) pathway [NAc, plaque-forming cells (PFC) and VTA] in many of the same BXD strains used in this study (Wolen *et al.* 2012). Therefore, to identify genes potentially functional in nicotine place conditioning we correlated these brain regional expression patterns to the transformed place conditioning behavioral data (nicotine–saline) across the BXD

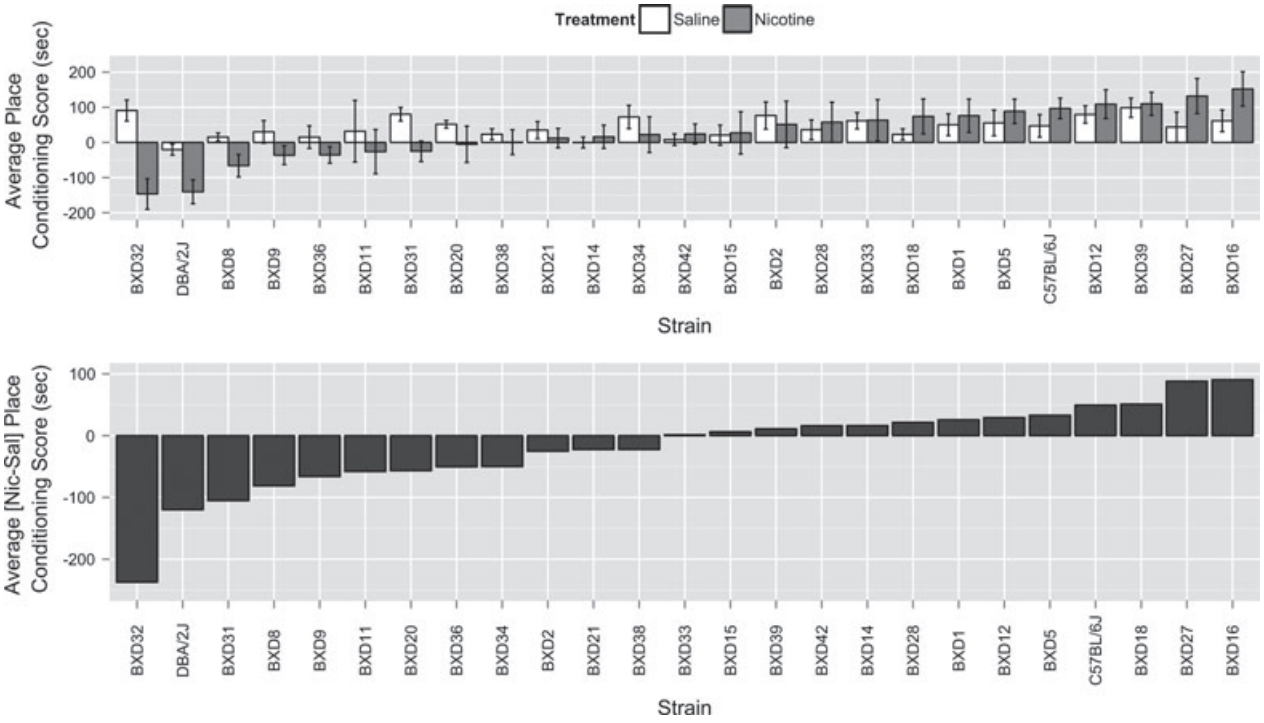

Figure 1: BXD strain distribution for place conditioning for 0.5 mg/kg nicotine. (a) Following conditioning with either saline–saline (0.9%) or saline–nicotine (0.5 mg/kg), nicotine place conditioning scores (black) on test day for BXD strains follow a continuous distribution, indicative of a quantitative trait. Progenitor strains, C57BL/6J and DBA/2J, demonstrate divergent phenotypes for this trait. Each point represents the mean ± SEM of $n = 6–12$ mice per group. (b) Transformed distribution, nicotine minus saline BXD strain means.

Table 1: BXD place preference scores correlate inversely to the number of striatal cholinergic neurons

GeneNetwork ID	Phenotype	Authors	Spearman's rho	*n* Strains	*P*(rho)
10106	Central nervous system, morphology: striatum cholinergic neurons, section 11 [*n* neurons/section]	Dains *et al.* 1996	−0.7356738	18	0.000268
10107	Central nervous system, morphology: striatum cholinergic neurons, section 14 (*n* neurons/section)		−0.5443756	18	0.0180843
10110	Central nervous system, morphology: striatum cholinergic neurons, section 22 (*n* neurons/section)		−0.6417141	18	0.0032019

Within multiple sections of the striatum, the number of cholinergic neurons (Dains *et al.* 1996) is significantly inversely correlated with nicotine place preference scores of BXD mice on day 5 of the CPP paradigm. All genetic correlations are Spearman correlations performed using GeneNetwork.
GN ID, GeneNetwork Record ID; *n*, number of BXD strains used for correlations.

strains. This identified 2044, 2099 and 1253 probesets in NAc, PFC and VTA, respectively, demonstrating provisional correlations ($P < 0.05$; see Tables S4–S6) with nicotine place conditioning. Further analysis of these expression correlates will be reported elsewhere, but for the purposes of this report, we focused the data analysis by surveying these results for nicotinic receptor-related genes that also contained putative *cis* expression QTL (eQTL). Such *cis* eQTL, resulting most frequently from genetic variation within or near the gene itself, have a larger effect size, and are primary genetic drivers of variation, whereas *trans* eQTL have secondary or tertiary effects (Drake *et al.* 2006; Schadt *et al.* 2003). We proposed that this might implicate specific nicotinic receptors not previously identified as modulating nicotine place conditioning. This analysis revealed only *Chrna7* in NAc containing a putative *cis* eQTL and its expression significantly correlated with normalized nicotine place conditioning phenotype (see Table S7). Figure 2 depicts correlations between the normalized phenotype and *Chrna7* mRNA expression (Fig. 2a, probeset ID 1440681_at and Fig. 2b, probeset ID 1450229_at) in each of the three brain regions of the mesolimbic DA reward pathway (VCU BXD NA, VTA and PFC, RMA saline datasets). Although preference scores correlated significantly to *Chrna7* mRNA expression

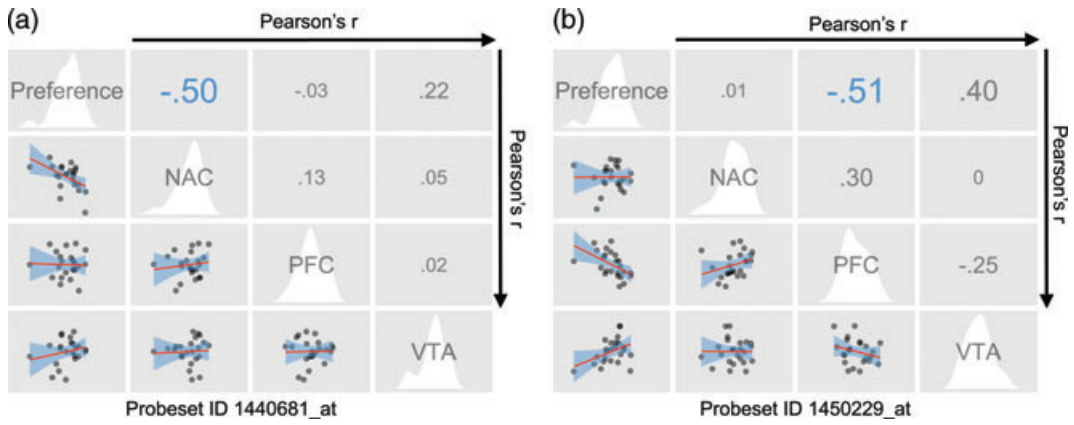

Figure 2: The nicotine place conditioning phenotype is genetically correlated to *Chrna7* basal mRNA expression in the NAc, but not the prefrontal cortex, or ventral midbrain. Conditioning scores (nicotine−saline) significantly correlate with basal *Chrna7* expression (*Chrna7* probeset ID = 1440681_at, panel (a) in the NAc and prefrontal cortex (*Chrna7* probeset ID = 1450229_at, panel (b), but not ventral midbrain (denoted as VTA). Correlation scattergrams (left of diagonal), univariate density plots (in white, along the diagonal) and Pearson's *r* values (right of diagonal) are displayed. For the correlation scattergrams, the linear fits are plotted in red with 50% confidence intervals in blue. Each point represents the mean for a BXD strain. A blue *r* value denotes a significant correlation, while gray *r* values are non-significant at an $\alpha = 0.05$. All expression data are saline RMA values from the VCU BXD NA, PFC and VMB. Datasets, with probes containing SNPs between B6 and D2 genotypes are removed.

in both NAc (Pearson's $r = -0.50$, $P = 9.04\text{E}{-3}$, probeset 1446081_at) and PFC (Pearson's $r = -0.51$, $P = 9.07\text{E}{-3}$, probeset 1450229_at), the putative *cis* eQTL only existed in the NAc for probeset 1446081_at, therefore we focused on this probeset for the remaining analyses. Together, these data suggest that genetic variation in *Chrna7* expression modifies nicotine place conditioning, with strains having low basal mRNA expression of *Chrna7* in the NAc being more susceptible to the reward-like properties of nicotine.

Figure 3a,b depicts a suggestive *Chrna7 cis* eQTL (probeset 1440681_at) present in the NAc across the BXD panel of mice. Following 2000 permutations, LOD scores of 2.35 and 3.77 denoted a suggestive or significant eQTL, respectively. These statistical limits are actually quite conservative as they reflect genome-wide corrections for multiple testing. As two probes within this probeset have single nucleotide polymorphisms (SNPs) between B6 and D2 mice that may have influenced complementary RNA (cRNA) hybridization to the oligonucleotide microarray, causing a false *cis* eQTL, we excluded those probes and re-performed interval mapping, which confirmed the putative *cis* eQTL (Fig. 3a,b). Microarray NAc data, showing higher *Chrna7* transcript levels in D2 mice compared to B6 mice, were validated by qRT-PCR for *Chrna7* from B6 and D2 NAc tissue samples ($^{*}P < 0.001$, $t[12] = 5.068$, $n = 7$/group, Student's *t*-test; Fig. 3c). The *Chrna7* gene is highly polymorphic between the B6 and D2 progenitor strains (285 SNPs; Table S8), thus expression differences may be attributed to one or more of these SNPs.

Knock-out of the α7 nAChR increases sensitivity to, whereas gain-of-function or agonism of the α7 nAChR abolishes the nicotine CPP phenotype in mice

We used a series of pharmacological and genetic manipulations in mice to confirm the involvement of the α7 nAChR in nicotine CPP as expected from the inverse correlation between basal *Chrna7* mRNA expression and nicotine CPP. First, α7 KO mice demonstrated significant preference for 0.1 mg/kg nicotine vs. saline treatment (one-way ANOVA, followed by Dunnett's *post hoc* vs. within-genotype saline group, $F_{[2,20]} = 16.8854$, $^{\#}P < 0.05$, $n = 7$–9/group). This dose of nicotine does not routinely produce place preference in C57BL/6J mice (Walters *et al.* 2006), suggesting an increase in sensitivity to the reward-like properties of nicotine in CPP. However, this difference in sensitivity between groups was not observed with 0.5 mg/kg of nicotine as both genotypes demonstrated equal amounts of CPP (one-way ANOVA, followed by Dunnett's *post hoc* vs. within-genotype saline $F_{KO[2,20]} = 16.8854$, $^{*}P_{KO} < 0.01$, $n_{KO} = 7$–8/group; $F_{WT[2,21]} = 24.0645$, $^{*}P_{WT} < 0.01$, $n_{WT} = 7$–8/group; Fig. 4a). Finally, the preference for 0.1 mg/kg nicotine was significantly lower than preference for 0.5 mg/kg nicotine in α7 KO mice (one-way ANOVA, followed by Tukey's HSD *post hoc* to compare doses, $F_{KO[2,20]} = 16.8854$, $^{\ddagger}P_{KO} < 0.01$, $n_{KO} = 7$–8/group). A two-way ANOVA evaluating nicotine doses (0, 0.1 and 0.5 mg/kg) and mouse genotype (WT and KO; $F_{[5,41]} = 16.9029$, $n = 7$–9/group) revealed significant main effects of both dose ($P < 0.01$) and genotype ($P < 0.05$), but no significant interaction between dose and genotype ($P = 0.3371$).

In contrast, gain-of-function, α7 KI mice did not develop preference to nicotine at either 0.1 or 0.5 mg/kg (one-way ANOVA, Tukey's HSD *post hoc* vs. within-genotype saline control, $F_{KI[2,29]} = 0.0080$, $P_{KI} = 0.9920$, $n_{KI} = 8$–16/group), but WT littermates developed normal preference for 0.5 mg/kg nicotine (one-way ANOVA, Tukey's HSD *post hoc* vs. within-genotype saline control, $F_{WT[2,21]} = 12.1665$, $^{*}P_{WT} < 0.01$, $n_{WT} = 8$/group; Fig. 4b). This follows the trend observed for low or no preference in BXD strains

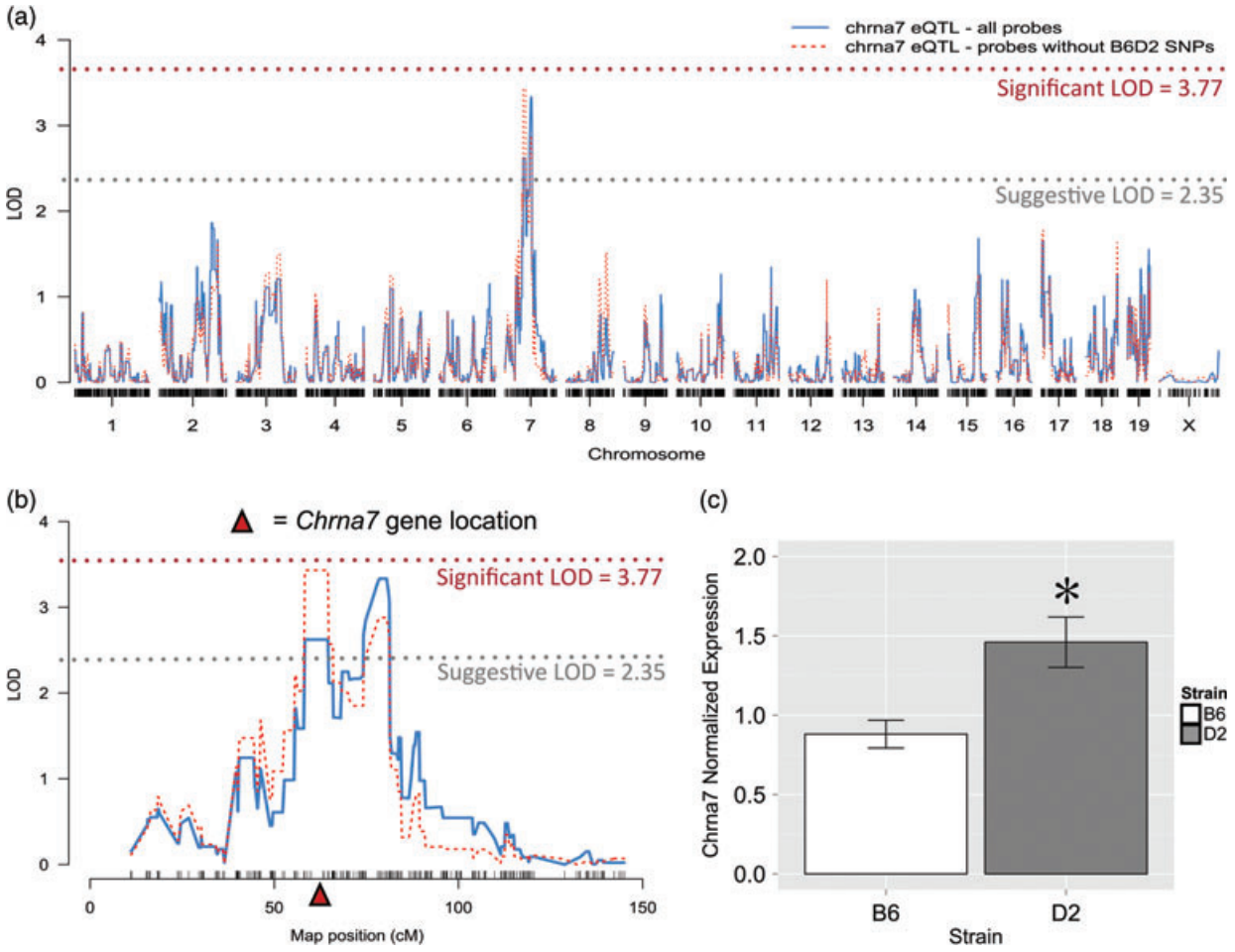

Figure 3: Genome-wide interval map for *Chrna7* mRNA levels across the BXD RI panel. (a) A suggestive *cis* expression QTL (blue solid line) exists on chromosome 7 for *Chrna7* mRNA levels in the NAc (VCU, BXD, NA, dataset saline, RMA values and probeset ID 1440681_at). The *cis* eQTL remained after removal of the probes containing SNPs between B6 and D2 mice (red dotted line). The DBA/2J genotype for *Chrna7* increases its expression. (b) An enlargement of chromosome 7 reveals two possible QTL peaks driving the mRNA expression of *Chrna7*, of which the proximal peak harbors *Chrna7* (at 70.24Mb). (c) qRT-PCR validation of microarray results. Basal mRNA expression of *Chrna7* in the NAc is significantly greater in D2 mice compared to B6 mice. Each point represents the mean ± SEM (*$P < 0.01$).

having high basal mRNA levels of *Chrna7* (Fig. 2). Finally, the α7-selective agonist, PHA-543613, was able to dose-dependently block preference in B6 mice for 0.5 mg/kg nicotine (one-way ANOVA, followed by Dunnett's *post hoc* vs. PHA0/Nic0.5, $F_{B6[3,25]} = 10.4663$, *$P_{B6} < 0.01$, $n_{B6} = 6–8$/group; Fig. 4c), a dose that routinely produces place preference in this strain of mice (Walters *et al.* 2006). The highest dose of PHA-543613 (12.0 mg/kg) did not significantly alter preference scores in B6 mice on its own (data not demonstrated, one-way ANOVA vs. vehicle, Tukey's HSD *post hoc*, $F_{B6[1,14]} = 0.5321$, $P_{B6} = 0.4778$, $n_{B6} = 8$/group). Furthermore, Fig. 4d reveals that blockade of nicotine preference by PHA-543613 can be reversed using 10.0 mg/kg of the α7-selective antagonist, MLA (one-way ANOVA, followed by Tukey's HSD *post* hoc vs. PHA12/Nic0.5 alone, $F_{B6[1,13]} = 46.5249$, *$P_{B6} < 0.01$, $n_{B6} = 7–8$/group). Also demonstrated and previously reported, this dose of MLA was unable to block preference for 0.5 mg/kg nicotine in B6 mice on its own (Walters *et al.* 2006).

To control for possible learning deficits or enhancements in the gene-targeted mice and to determine if this effect was specific to nicotine, we tested cocaine CPP in α7 WT, KI and KO mice. Mice of all three genotypes developed place preference similar to 10 mg/kg cocaine (one-way ANOVA, followed by Dunnett's *post hoc* vs. within-genotype saline, $F_{KO\text{-}a[1,13]} = 83.3130$, *$P_{KO\text{-}a} < 0.01$, $n_{KO\text{-}a} = 7–8$/group, $F_{WT\text{-}a[1,13]} = 47.3111$, *$P_{WT\text{-}a} < 0.01$, $n_{WT\text{-}a} = 7–8$/group, $F_{KI\text{-}b[1,16]} = 23.6439$, *$P_{KI\text{-}b} < 0.01$, $n_{KI\text{-}b} = 9$/group, $F_{WT\text{-}b[1,15]} = 13.8604$, *$P_{WT\text{-}b} < 0.01$, $n_{WT\text{-}b} = 8–9$/group; Fig. 5a,b), suggesting that not only these mice are responsive to pavlovian drug conditioning but also that the α7 mechanism is not applicable to another drug of

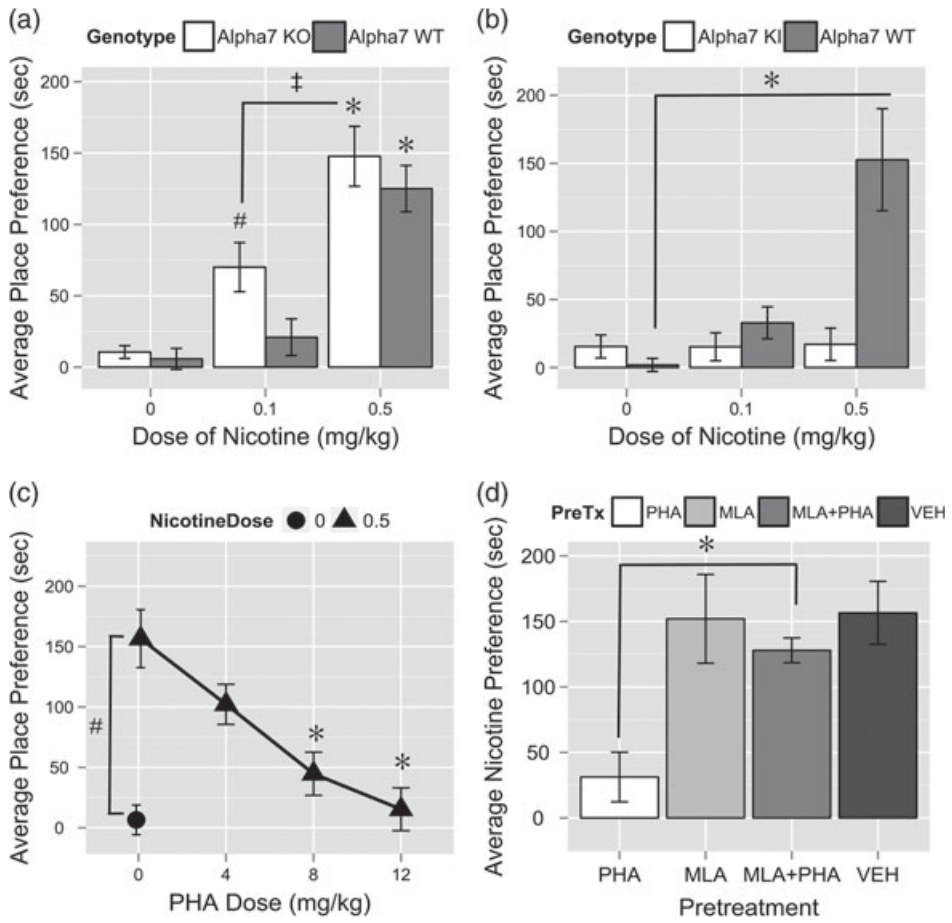

Figure 4: Deletion of the α7 nAChR results in increased sensitivity to nicotine place preference and knock-in or agonism of the α7 nAChR prevents nicotine place preference. Panel (a), a significant increase in place preference scores for 0.5 mg/kg nicotine was observed in both α7 KO and WT mice. α7 KO mice display place preference for 0.1 mg/kg nicotine; preference for 0.1 mg/kg nicotine was of significantly lower magnitude than preference for 0.5 mg/kg nicotine. Panel (b), only wild-type (WT) mice, but not α7 knock-in (KI) mice, demonstrate nicotine place preference for 0.5 mg/kg of nicotine. (c) Place preference for 0.5 mg/kg nicotine was significantly higher than preference for saline in B6 mice. Pretreatment with PHA-543613 dose-dependently blocked place preference for 0.5 mg/kg nicotine at 8.0 and 12.0 mg/kg PHA. (d) For CPP for 0.5 mg/kg of nicotine, 12.0 mg/kg PHA blocked preference; this was reversed by pretreatment with 10.0 mg/kg of methyllycacontinine (MLA). MLA alone did not alter nicotine preference. Each point represents the mean ± SEM (*/‡$P < 0.01$, #$P < 0.05$). Unless otherwise denoted, all symbols of significance denote comparisons for within-group vehicle treatment.

abuse, cocaine. As an additional control to rule out *Chrna7* involvement in place preference for cocaine, we attempted to block preference in C57BL/6J mice with PHA-543613, but saw no attenuation of cocaine place preference compared to control (one-way ANOVA, Dunnett's *post hoc* vs. within-group vehicle, $F_{B6[1,8]} = 23.0984$, $*P_{B6} < 0.01$, $n_{B6} = 5$/group, one-way ANOVA, Dunnett's *post hoc* vs. within-group PHA, $F_{B6[1,14]} = 4.9642$, $\#P_{B6} < 0.05$, $n_{B6} = 8$/group; Fig. 5c).

Genetic interactions between Chrna7 and insulin-related genes in the NAc may contribute to preference for nicotine

In order to explore possible mechanisms underlying the enhanced sensitivity to nicotine observed in α7 KO mice with the CPP test, contrasted to their WT counterparts, we performed microarray analysis on NAc samples from only α7 KO and WT mice. Following statistical analyses and filtering, differentially regulated genes revealed the top significant network ($P = 1.0E{-}49$) as containing multiple genes involved in insulin signaling (Fig. 6a). We observed that in α7 KO mice, expression of the insulin growth factor binding proteins, *Igfbp2* and *Igfbp6*, were significantly increased, but insulin-degrading enzyme (*Ide*) expression was significantly decreased in the NAc compared to WT mice.

Finally, qRT-PCR performed from α7 KO and WT NAc samples confirmed significant downregulation of *Ide* mRNA ($P < 0.05$, $t[7] = 2.957$), upregulation of *Igfbp6* mRNA ($P < 0.05$, $t[7] = 2.230$), and a non-significant trend for

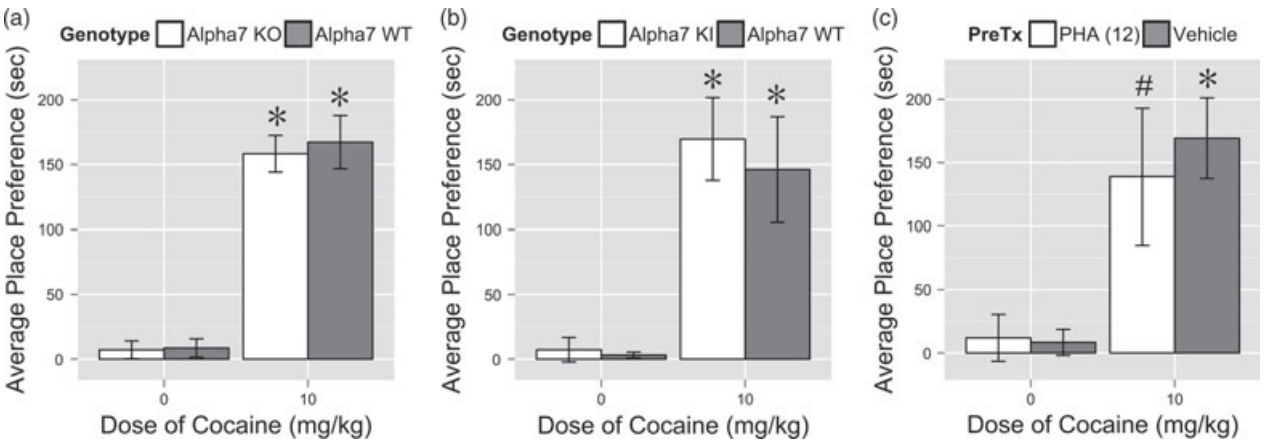

Figure 5: α7 knock-in and knock-out mice develop normal place preference to a 10 mg/kg dose of cocaine; **pretreatment with PHA in C57BL/6J mice does not alter cocaine place preference**. Panels (a) and (b), a significant increase in place preference scores for 10 mg/kg cocaine compared to within-genotype saline treatment was observed for all genotypes tested. Panel (c), C57BL/6J mice develop place preference to 10 mg/kg cocaine. Pretreatment with PHA did not block cocaine place preference in C57BL/6J mice. Each point represents the mean ± SEM (*$P < 0.01$, #$P < 0.05$).

differential regulation of *Igfbp2* ($P = 0.11$, $t[7] = 1.07$; see Table 2). Furthermore, within the BXD panel of mice, we identified a co-expression network of *Chrna7* and insulin-related genes in the NAc, which are significantly genetically correlated to each other as well as the transformed nicotine preference phenotype, suggesting novel and complex gene–gene interactions underlying place preference to nicotine (Fig. 6b). The expression networks and qRT-PCR data suggest increased insulin signaling in both α7 KO and BXDs with low NAc levels of *Chrna7* mRNA, thus perhaps suggesting a regulatory interaction.

Knock-out of the α7 nAChR results in basal increases in insulin signaling in the NAc

To confirm functional differences in insulin signaling in the NAc of α7 KO and WT mice, we performed immunoblotting for proteins known to be involved in insulin signaling (Fig. 7). In contrast to our microarray data in which *Ide* mRNA was decreased in the NAc of α7 KO mice, we found that IDE protein was significantly upregulated compared to WT (*$P < 0.01$, $t[6]_{IDE} = 5.18$). Additionally, we found a trend for decreased total insulin receptor levels, INSR-β (intracellular subunit) ($P = 0.113$, $t[6]_{INSR} = 1.87$) and significantly increased phosphorylation of multiple sites of the INSR in α7 KO mice compared to WT mice ($t[9]_{pIR(Y1158,\ Y1162,\ Y1163)/IR} = 4.60$, $t[9]_{pIR(Y932)/IR} = 4.49$), Student's *t*-test, $n_{KO} = 6$, $n_{WT} = 5$.

Discussion

In this study, we combined behavioral and expression profiling across a genetic reference panel to identify genes underlying nicotine's reward-like behavioral response in mice. This implicated *Chrna7* as a candidate gene. Interestingly, previous mouse studies have linked *Chrna7* polymorphisms with strain differences in neuronal expression (Stitzel *et al.* 1996) and distribution of α7 nAChRs (Adams *et al.* 2001), as well as behaviors such as nicotine seizure sensitivity (Stitzel *et al.* 1998). However, until now, the role of *Chrna7* in nicotine CPP had not been rigorously evaluated. In this study, we verified the involvement of *Chrna7* in nicotine CPP through the use of behavioral genetic studies in BXD mice, gene-targeted mice and pharmacological tools. By extending our genomic analysis in α7 KO mice, we provide evidence that an insulin gene expression network in the NAc, regulated by *Chrna7*, may contribute to reward-like effects of nicotine CPP.

Using the BXD panel of mice, we found that basal *Chrna7* mRNA expression in the NAc was significantly negatively correlated to place conditioning for nicotine, despite the lack of robustly significant behavioral QTL for nicotine place conditioning in the vicinity of *Chrna7*. This discrepancy might be accounted for by a number of factors, such as low heritability of nicotine preference coupled with the number of strains studied in these experiments, the influence of non-genetic factors influencing intrastrain and interstrain variance in this phenotype, as well as the complexity of the behavioral assay. Furthermore, many genes or loci of small genetic effect underlying the place conditioning behavior, rather than one or few loci of large effect would likely mask detection of a significant behavioral QTL with the number of strains assayed herein. Studies with the α7 agonist, PHA-543613, dose-dependent attenuation of CPP and this was reversed by the α7 antagonist, MLA. α7 KI mice did not display nicotine CPP, but conversely, α7 KO mice were more sensitive to a low dose of nicotine, suggesting an increased perceived reward-like effect in these mice. Together, these behavioral data strongly implicate *Chrna7* as a candidate gene modulating nicotine CPP reward-like phenotypes.

Previous studies using α7 KO mice or an α7 antagonist in rats have reported that in contrast to the β2-containing nAChR, the α7 nAChR is not required for nicotine CPP

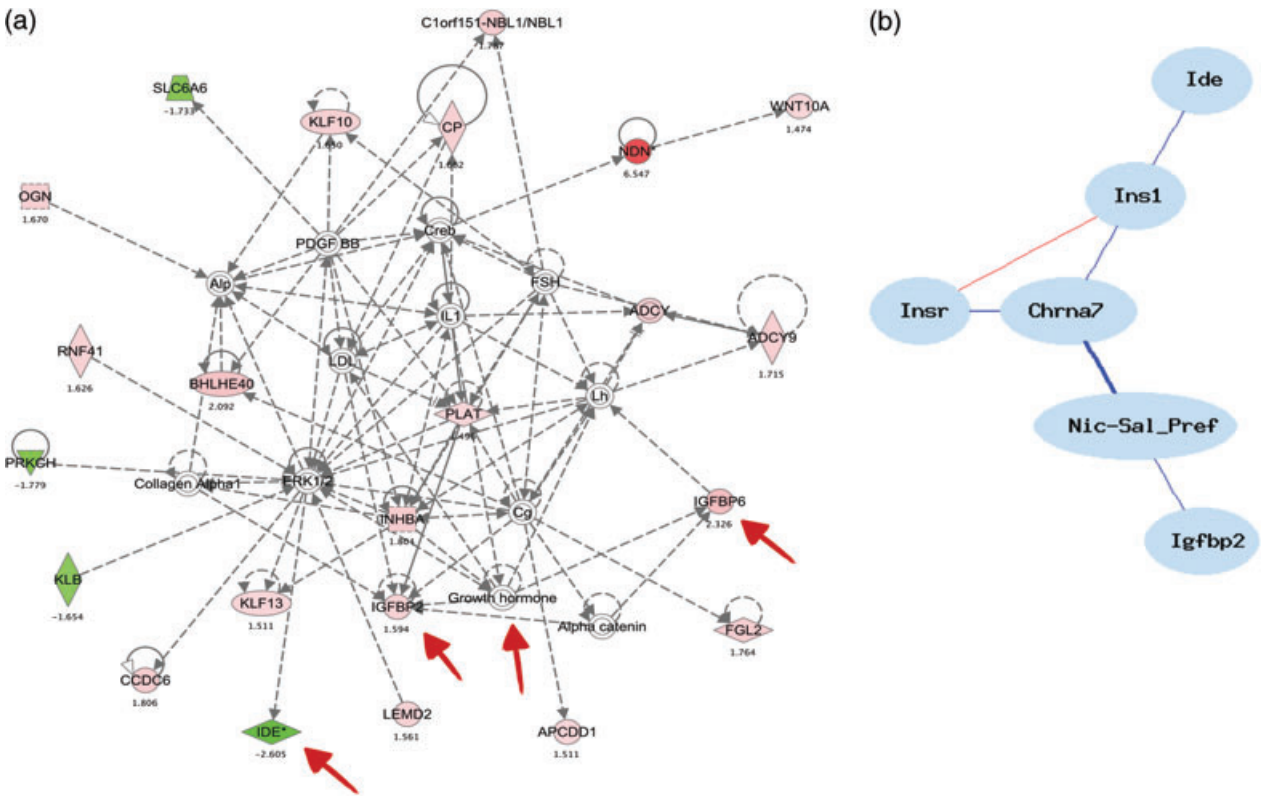

Figure 6: Knock-out of the α7 nAChR results in alterations to an insulin-related gene network. (a) Top-ranked biological network of genes differentially regulated in the NAc between α7 KO and WT mice (red, upregulated; green, downregulated; colorless, imputed gene; number below each gene, KO/WT s-score; and red arrows, insulin-related genes). (b) Genetic correlation network performed using BXD mRNA expression data, displaying co-regulation of *Chrna7* and multiple insulin-related genes in the NAc. (All correlations drawn are significant and used Pearson's *r*, red, positive; blue, negative; bold, $r \geq |0.5|$; solid, $|0.41| \geq r < |0.49|$).

Table 2: Quantitative RT-PCR confirms differential insulin-related gene expression in the NAc of α7 KO and WT mice

Gene	Normalized relative KO expression	KO SEM	Normalized relative WT expression	WT SEM	*P*-value
Ide	0.731	0.091	1.102	0.096	1.55E−02
Igfbp2	1.768	0.249	1.166	0.166	1.08E−01
Igfbp6	1.549	0.144	1.071	0.136	3.58E−02
Ndn	2.374	0.160	1.177	0.078	3.09E−03

Basal mRNA expression of *Ide* is significantly downregulated in the NAc of KO mice compared to WT mice, whereas *Igfbp6* is significantly upregulated, and there is a trend toward upregulation of *Igfbp2*. Each point represents the mean of ± SEM ($n_{KO} = 4$ and $n_{WT} = 5$). All genes were normalized to the housekeeping gene, *Gapdh*.

(Walters *et al.* 2006) or self-administration (Brunzell & McIntosh 2012), respectively. Additionally, it was reported that reductions in α7 nAChR activity because of antagonist administration into the NAc shell, as well as anterior cingulate cortex, dose-dependently increased the motivation of rats to self-administer nicotine (Brunzell & McIntosh 2012), suggesting that lower α7 nAChR activity may increase the intake of nicotine. By surveying the BXD panel and including a lower dose of nicotine in α7 KO mice, our studies have unmasked the first evidence that *Chrna7* transcript levels in the NAc are both genetically regulated and may be an important factor in determining the magnitude of nicotine's reward-like effects as measured by CPP. Although CPP and self-administration were originally thought to be isomorphic models of drug reward, it is now commonly accepted that self-administration models reinforcement, and CPP models 'reward' (and is influenced by other factors such as memory). Thus, these assays may measure overlapping as well as distinct components of drug-seeking behavior in animals (Bardo & Bevins 2000). As the CPP test measures contextual cues associated with a drug's perceived 'reward', our data suggest that lower levels of *Chrna7* mRNA in the NAc

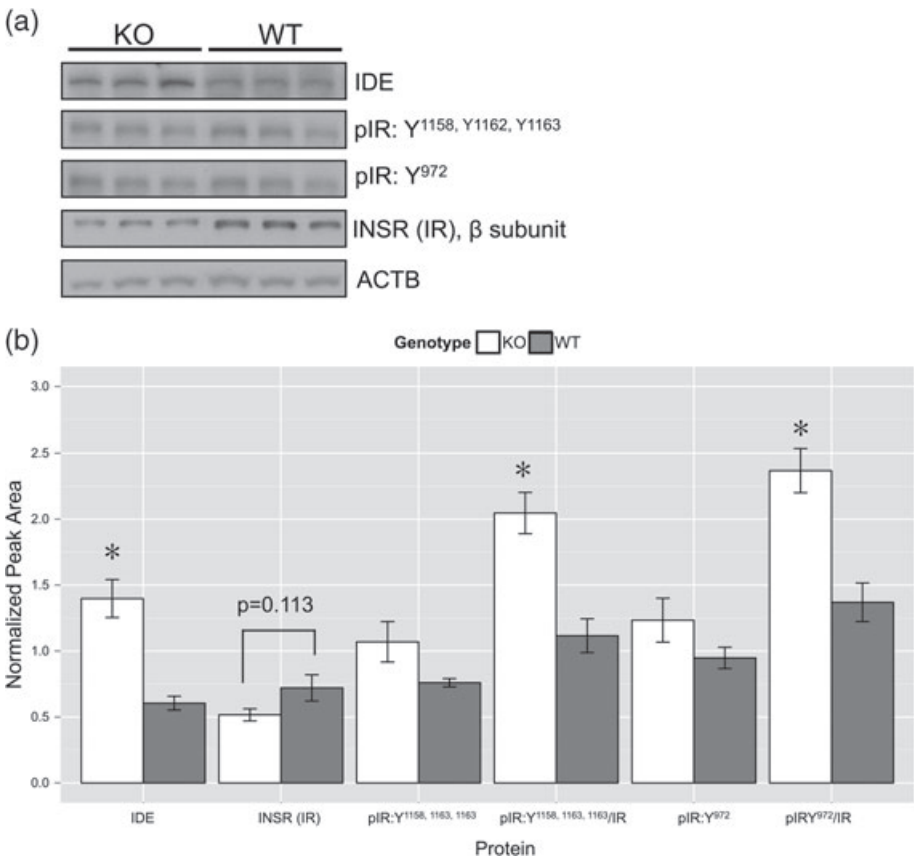

Figure 7: α7 knock-out mice display differential basal expression of insulin-related proteins in the nucleus acumbens. (a) Representative immunoblots of NAc samples from individual mice. IDE, insulin-degrading enzyme; pIR, phosphorylated insulin receptor (Y, tyrosine residue phosphorylated); INSR/IR, β subunit of the insulin receptor; ACTB, β-actin. (b) Quantitation and statistical analyses of immunoblot samples ($n_{KO} = 6$, $n_{WT} = 5$) revealed that IDE protein levels as well the degree of phosphorylation of the total insulin receptor were significantly increased in α7 KO mice compared to WT mice, while total insulin receptor levels demonstrated a trend for being decreased compared to WT mice. All proteins were normalized to ACTB (*$P < 0.01$).

contributed to an altered neural response to cue-related behaviors, allowing nicotine-seeking behavior to persist.

Using microarray analysis, we discovered that gene-targeting of the α7 nAChR results in disruption of gene expression for an insulin-signaling network in the NAc (Fig. 6a). Independently, *Chrna7* expression was genetically correlated with a network of insulin-related genes in the NAc across the BXD panel (Fig. 6b), strongly suggesting that our microarray results likely were not because of developmental compensation in α7 KO mice. Insulin-degrading enzyme mRNA was significantly decreased in α7 KO mice, and previous rodent studies have demonstrated that functional disruption of this gene, results in hyperinsulinemia and glucose intolerance (Fakhrai-Rad *et al.* 2000; Farris *et al.* 2003). Additionally, *Ide* mRNA expression is altered in rats following nicotine treatment (Polesskaya *et al.* 2007), whereas an allele of the *Ide* gene is associated with plasma cotinine levels in both European and African American smokers (Hamidovic *et al.* 2012). Together with the present study, these data implicate a possible role of *Ide* in nicotine addiction. Based on these studies, we predicted that in the NAc, α7 KO mice should have increased insulin signaling compared to WT mice.

Insulin and insulin growth factor 1 (IGF1) are produced peripherally and can readily cross the blood brain barrier *via* an active transport system to elicit endocrine signaling events. IGF1 is also secreted locally by neuronal cells (neurons, microglia and astrocytes) and participates in paracrine signaling within the brain (Russo *et al.* 2005). Insulin and IGF1 signal through either of their receptors, INSR (insulin receptor) or IGF1R (IGF1 receptor), or through a hybrid receptor formed from dimerization of the INSR and IGF1R receptors (Russo *et al.* 2005). Activation of the receptor can trigger two canonical signaling pathways, PI3K-AKT and Ras-ERK. In rat NAc slices, insulin potentiates cocaine-induced DA release and this was reversed using a PI3K inhibitor (Schoffelmeer *et al.* 2011). Additionally, insulin receptor activation in rat striatal cultures leads to increases in DAT mRNA expression and transporter function that resulted in increased DA reuptake (Patterson *et al.* 1998). Insulin regulation of DAT was blocked in cell culture with a PI3K inhibitor, providing further evidence for the PI3K-AKT pathway in insulin's modulation of DA uptake (Carvelli *et al.* 2002). These studies suggest a negative feedback loop in which insulin promotes both DA release and DA reuptake. Interestingly, α7 KO mice have been shown to have significantly higher levels of and longer persistence of nicotine-induced DA release in the NAc compared to WT mice (Besson *et al.* 2012). This enhancement of DA release within the brain's reward center in α7 KO mice could contribute to increased sensitivity to low dose nicotine observed in the CPP test.

Our studies also provide evidence of altered insulin signaling in α7 KO mice through multiple genes and proteins.

Ide transcript levels in α7 KO mice are lower (Table 2) and IDE protein levels are higher compared to WT mice (Fig. 7b). Additionally, we found significantly higher activation of the INSR as well as a trend for decreased INSR protein levels in α7 KO mice (Fig. 7). Furthermore, our microarray experiment revealed that α7 KO mice, compared to WT mice, had higher NAc transcript levels of genes for two insulin growth factor binding proteins, *Igfbp6* and *Igfbp2*, which regulate IGF1 bioavailability.

Taken together, our studies provide evidence that *Chrna7* modulates nicotine reward. Our genomic and molecular studies suggest that Chrna7 influences an expression network of insulin-signaling genes and their proteins which may alter downstream DA signaling and lead to altered nicotine reward-like behaviors. These studies are the first to elucidate the genetic interplay between *Chrna7* and insulin signaling in the NAc and a role for such interactions in nicotine behavior. Future studies will directly test these relationships between nicotine CPP, *Chrna7*, and the insulin signaling pathway in the NAc. These genetic and protein interactions have implications for uncovering new therapeutic targets for nicotine cessation pharmacotherapies.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Appendix S1. An experimental timeline, details of mouse handling and husbandry, as well as CPP calculation details.

Table S1: Locomotor activity counts on test day. Locomotor strain mean comparisons between vehicle and drug groups for multiple drugs tested revealed that there were no significant differences between vehicle and drug groups for cocaine, PHA-543613 and MLA within any genotype of mice tested. Except for BXD strains 27, 38 and 8, there were no within-strain differences in locomotor activity counts on test day for mice previously treated with saline or nicotine. Across the BXD panel, there was no significant effect of nicotine on locomotor activity on test day.

Table S2: Intraclass correlation coefficients (ICC) of BXD nicotine and saline CPP phenotypes. Intraclass correlation coefficients with confidence intervals for saline and nicotine phenotypes are listed as measures of heritabilities of these traits across the BXD panel of mice. Saline place preference and post-conditioning scores ICCs were near zero, suggesting that treatment of saline does not genetically influence these phenotypes. Nicotine place preference and post-conditioning ICC estimates were 19% and 13%, respectively, with upper confidence levels reaching 33% and 25%. Locomotor activity ICC estimates ranged from 12% to 40%. Removal of BXD strains 27, 32 and 8 did not significantly change heritabilities for these phenotypes.

Table S3: Nicotine place preference correlates to other BXD published phenotypes. Spearman correlations between

nicotine–saline place preference in the BXD panel and other published BXD phenotypes were performed using GeneNetwork. All records with females and with fewer than 10 strains in common in our data were excluded and only records with $P(\text{rho}) < 0.05$ are listed in the table.

Table S4: Nicotine place preference phenotype correlations with probesets in the nucleus accumbens. Pearson correlations between nicotine post-conditioning scores and nucleus accumbens gene expression data (VCU NA dataset (Wolen *et al.* 2012)) were calculated using GeneNetwork. The list was filtered for correlation P values < 0.05 and contained 2004 significantly correlated probesets.

Table S5: Nicotine place preference phenotype correlations with probesets in the prefrontal cortex. Pearson correlations between nicotine post-conditioning scores and nucleus accumbens gene expression data (VCU PFC Dataset (Wolen *et al.* 2012)) were calculated using GeneNetwork. The list was filtered for correlation P values < 0.05 and contained 2099 significantly correlated probesets.

Table S6: Nicotine place preference phenotype correlations with probesets in the ventral midbrain. Pearson correlations between nicotine post-conditioning scores and nucleus accumbens gene expression data (VCU VTA dataset (Wolen *et al.* 2012)) were calculated using GeneNetwork. The list was filtered for correlation P values < 0.05 and contained 1253 significantly correlated probesets.

Table S7: Nicotine place preference phenotype correlations with *cis* probesets in the BXD nucleus accumbens. Pearson correlations between nicotine post-conditioning scores and nucleus accumbens gene expression data (VCU NA dataset (Wolen *et al.* 2012)) were calculated using GeneNetwork. This list was filtered for correlation P values < 0.05, LRS ≥ 13.8 (LOD ≥ 3.0), and probesets with putative *cis* eQTL in the nucleus accumbens, which revealed 336 probesets.

Table S8: Genetic variation in the *Chrna7* gene between C57BL/6J and DBA/2J mice. Single nucleotide polymorphisms between B6 and D2 mice that may account for the *cis* eQTL are listed in this table. One or more of these SNPs may contribute to differential transcript levels of the Chrna7 in the NAc observed between B6 and D2 mice (Table adapted from GeneNetwork).

Table S9: Significantly differentially regulated genes in the NAc between $\alpha 7$ KO and WT mice. Following a SAM analysis with a $\delta = 0.314$ and an FDR $= 9.802\%$, 391 genes were found to be significantly differentially regulated in the NAc of $\alpha 7$ KO and WT mice. After filtering for s-scores $\geq |1.5|$, 108 probesets, 89 positively regulated and 19 negatively regulated, were found.

Table S10: Primer sequences used for quantitative, real-time PCR. Table containing all primer sequences, Tms and amplicon sizes from the qRT-PCR experiments.

Table S11: Antibodies used for immunoblotting. Table containing all primer antibody catalog numbers, molecular weights, dilutions and incubation times used for immunoblotting.