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RESEARCH PAPER

The α3β4* nicotinic ACh receptor subtype mediates physical dependence to morphine: mouse and human studies

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BACKGROUND AND PURPOSE

Recent data have indicated that α3β4* neuronal nicotinic (n) ACh receptors may play a role in morphine dependence. Here we investigated if nACh receptors modulate morphine physical withdrawal.

EXPERIMENTAL APPROACHES

To assess the role of α3β4* nACh receptors in morphine withdrawal, we used a genetic correlation approach using publically available datasets within the GeneNetwork web resource, genetic knockout and pharmacological tools. Male and female European-American (n = 2772) and African-American (n = 1309) subjects from the Study of Addiction: Genetics and Environment dataset were assessed for possible associations of polymorphisms in the 15q25 gene cluster and opioid dependence.

KEY RESULTS

BXD recombinant mouse lines demonstrated an increased expression of α3, β4 and α5 nACh receptor mRNA in the forebrain and midbrain, which significantly correlated with increased defecation in mice undergoing morphine withdrawal. Mice overexpressing the gene cluster CHRNA5/A3/B4 exhibited increased somatic signs of withdrawal. Furthermore, α5 and β4 nACh receptor knockout mice expressed decreased somatic withdrawal signs compared with their wild-type counterparts. Moreover, selective α3β4* nACh receptor antagonists, α-conotoxin AuIB and AT-1001, attenuated somatic signs of morphine withdrawal in a dose-related manner. In addition, two human datasets revealed a protective role for variants in the CHRNA3 gene, which codes for the α3 nACh receptor subunit, in opioid dependence and withdrawal. In contrast, we found that the α4β2* nACh receptor subtype is not involved in morphine somatic withdrawal signs.

CONCLUSION AND IMPLICATIONS
Overall, our findings suggest an important role for the α3β4* nACh receptor subtype in morphine physical dependence.

Abbreviations
AuIB, α-conotoxin AuIB; KO, knockout; nACh receptors, nicotinic ACh receptors; WT, wild-type

Introduction

Opiate addiction from long-term use of prescription analgesics and illicit substances remains a major public health problem not only in the United States but around the world (Manchikanti *et al.*, 2012). An important component of opiate dependence is withdrawal, an aversive syndrome that occurs upon cessation of drug use and constitutes a powerful motivator for addicted individuals to continue to use drugs, even in the face of adverse consequences. The withdrawal syndrome is characterized in opioid-dependent humans as extremely unpleasant and manifests as tachycardia, hypertension, sweating, diarrhoea, vomiting, irritability, anxiety, shakes and insomnia. Current treatment for opiate withdrawal, which includes maintenance therapy with replacement opiates, methadone or buprenorphine, suffers from serious limitations. These limitations consist of dependence liability and withdrawal upon abrupt cessation (Kuhlman *et al.*, 1998; Dyer *et al.*, 1999). Therefore, the development of novel non-opioid pharmacotherapies for opiate withdrawal is critical.

A growing body of evidence supports the argument that neuronal nicotinic receptors (nACh receptors) may play an important role in opiate withdrawal. Recent rodent studies demonstrated that the administration of nicotinic antagonists attenuates signs of morphine withdrawal. For example, the non-selective nicotinic antagonists, mecamylamine and bis (2, 2, 6, 6-tetramethyl-4-piperidinyl) sebacate (BTMPS), were found to attenuate both spontaneous and naloxone-precipitated somatic signs in rats undergoing morphine withdrawal (Taraschenko *et al.*, 2005; Hall *et al.*, 2011). One particular nACh receptor subtype that has been indicated in decreasing somatic signs in precipitated morphine withdrawal is the α3β4* nACh receptor subtype (where * denotes the possible inclusion of additional subunits, see Alexander *et al.*, 2013). However, these antagonists, including dextromethorphan, bupropion and 18-methoxycoronaridine (18-MC), have poor or partial selectivity for the α3β4* nACh receptor subtype and produced variable levels of decrease in the somatic manifestations of naloxone-precipitated morphine withdrawal in rats (Rho and Glick, 1998; Panchal *et al.*, 2005; Taraschenko *et al.*, 2005). Altogether, these results imply a role for the α3β4* nACh receptor subtype in morphine withdrawal. However, the conclusions remain limited because the selectivity issues of drugs used.

The α3β4* nACh receptor subtype is an interesting candidate since the 15q25 gene cluster, which contains the CHRNA5/CHRNA3/CHRNB4 genes, coding for the α5, α3 and β4 nACh receptor subunits, respectively, has emerged as a candidate region contributing to risk of heavy smoking, nicotine dependence and smoking-related diseases in humans (Bierut, 2009). Rodent studies have also confirmed an important role for α5, α3 and β4 nACh receptor subunits in nicotine withdrawal and aversion (Wada *et al.*, 1990; Salas *et al.*, 2004a,b; 2009; Jackson *et al.*, 2010; Frahm *et al.*, 2011). The α5 nACh receptor subunit can co-assemble with the α3β4* nACh receptor subtype to form functional receptors in the peripheral ganglia, as well as centrally, in the medial habenula (MHb) and interpeduncular nucleus (IPN; Wada *et al.*, 1990; Zoli *et al.*, 1995; Quick *et al.*, 1999; Whiteaker *et al.*, 2002). These brain regions were recently reported to be involved in nicotine withdrawal and intake (Salas *et al.*, 2009; Fowler and Kenny, 2012).

This attractive hypothesis prompted us to investigate whether: (i) the α3β4* nACh receptor subtype plays a role in morphine physical dependence in mice; and (ii) the possible association of polymorphisms in human CHRNA5/CHRNA3/CHRNB4 genes with morphine dependence and withdrawal exists. To test the hypothesis that α3β4* receptors are involved in morphine somatic withdrawal, we used genetic correlation analyses across the BXD recombinant inbred (RI) mouse panel, selective α3β4* nACh receptor antagonists and finally transgenic mice with gene deletions or overexpression to elucidate the contribution of the α5, α3 and β4 nACh receptor subunits to morphine physical withdrawal. Lastly, human genetic association analyses were conducted using the Study of Addiction: Genetics and Environment (SAGE) European-American and African-American datasets to determine if variants in the 15q25 gene cluster are associated with opioid dependence.

Methods

All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). In addition, all receptor and drug nomenclature conforms to *British Journal of Pharmacology*'s Concise Guide to Pharmacology (Alexander *et al.*, 2013).

Animals

Male adult C57BL/6J mice were purchased from Jackson Laboratories. Mice null for the α5 (Jackson Laboratories, Bar Harbor, ME, USA), α4 and β2 (Institut Pasteur, Paris, France) nACh receptor subunits and their wild-type (WT) littermates were bred in an animal care facility at Virginia Commonwealth University (Richmond, VA). β4 nACh receptor knockout (KO) mice were generated and bred at Baylor College of Medicine (Houston, TX, USA) as described previously (Xu *et al.*, 1999). α5, α4, β2 and β4 mice were backcrossed at least 8 to 12 generations to C57BL/6J mice (Jackson Laboratories). Mutant/transgenic and WT littermates were obtained from crossing heterozygous mice. Transgenic mice overexpressing the

human cluster CHRNA5/A3/B4 (TgCHRNA5/A3/B4) (Gallego *et al.*, 2012) were obtained at Barcelona Biomedical Research Park (PRBB, Barcelona, Spain) by crossing transgenic mice with hybrid B6/SJL-F1 female mice (F1–F5). The non-transgenic littermates obtained from crosses of TgCHRNA3/A5/B4 mice and B6/SJL-F1J females served as controls. These transgenic mice bear increased [^{125}I]-epibatidine + 5I-A-85380 binding sites in brain regions where α3β4* is endogenously expressed and are hypersensitive to high doses of nicotine (Gallego *et al.*, 2012). Animals were 8–12 weeks of age at the start of the experiments and were group-housed (three to five per cage) under a 12 h light/dark cycle in a 21°C humidity-controlled AAALAC-approved animal care facility with *ad libitum* access to food and water. Experiments were performed during the light cycle and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University, Baylor College of Medicine, and the PRBB ethical committee.

General chronic morphine administration and precipitated withdrawal protocol

Mice were injected with saline or morphine s.c. over the course of 8 days as follows: days 1 and 2, mice received 25 mg·kg^{-1} morphine 2× a day. Days 3 and 4, mice received 50 mg·kg^{-1} morphine 2× a day. Days 5 and 6, mice received 80 mg·kg^{-1} morphine 2× a day. Days 7 and 8, mice received 100 mg·kg^{-1} 2× a day. On Day 9, mice were injected with 100 mg·kg^{-1} morphine in the morning. Two hours after morphine injection, mice were injected with naloxone (1 mg·kg^{-1}, s.c.) to precipitate somatic signs. Somatic signs were observed for 30 min immediately following naloxone injection.

Mice were individually placed in Plexiglass cages and were observed and scored for the manifestation of different withdrawal signs including the total number of jumps, wet dog shakes, paw tremors, backing, ptosis and diarrhoea. Results were reported as the average of the total signs per group. All testing was conducted in a blind manner.

Antagonist administration for morphine-precipitated withdrawal protocol

For groups receiving the antagonist AuIB via i.c.v., mice underwent the chronic morphine administration protocol as described in the previous section. However, on the evening of day 8, approximately 2 h after the last morphine injection, mice were anaesthetized with sodium pentobarbital (45 mg·kg^{-1} i.p.), and a scalp incision was made to expose the bregma. During anesthesia, mice were evaluated for pain with toe pinch prior to the procedure and checked every 3–5 min. Unilateral injection sites were prepared using a 26-gauge needle with a sleeve of polyurethane tubing to control the depth of the needle at a site 2 mm rostral and 2 mm lateral to the bregma at a depth of 2 mm. Animals were sutured, leaving the injection site accessible with gentle displacement of the scalp to enable an injection volume of 5 μL. Mice were allowed to recover overnight. On day 9, mice were injected with 100 mg·kg^{-1} morphine in the morning. Two hours after morphine injection, mice were injected i.c.v. with either vehicle or AuIB (1.75 or 3.5 pmol). Five minutes after AuIB or vehicle treatment, mice were injected with naloxone (1 mg·kg^{-1}, s.c.) to precipitate somatic signs. Somatic signs were observed for 30 min immediately following naloxone injection.

At the end of the study, a subset of animals were injected i.c.v. with 5 μL of cresyl violet dye 10 min before a lethal overdose of 65 mg·kg^{-1} sodium pentobarbital and perfused to confirm drug diffusion from the injection site to the lateral ventricle. Brain slices were collected, and mice were observed to have blue cresyl violet dye in both lateral ventricles.

For groups treated with AT-1001, on day 9, 2 h after morphine injection, mice were injected i.p. with vehicle or AT-1001 (1 or 3 mg·kg^{-1}) and, 15 min later, with naloxone. Somatic signs were observed for 30 min immediately following naloxone injection as described above.

To assess the role of α4β2* nACh receptors in morphine withdrawal, in a separate group, mice received the same morphine protocol as described in the previous section and 2 h after morphine injection on withdrawal day, mice were injected s.c. with vehicle or DHβE (3 mg·kg^{-1}). Five minutes after DHβE or vehicle treatment, mice were injected with naloxone (1 mg·kg^{-1}, s.c.) to precipitate somatic signs. Somatic signs were observed following naloxone injection as described above.

A similar protocol of morphine-precipitated withdrawal was followed with studies using α5, α4, β2 and β4 nACh receptor KO mice and their WT counterparts. Control groups treated with chronic saline were also assessed. Each treatment group contained n = 6–8 mice.

A slightly different administration protocol was used to investigate morphine dependence in the TgCHRNA5/A3/B4 model due to the different genetic background used (hybrid C57/SJL strain). Since WT mice showed a ceiling effect with the previously described protocol (data not shown), we reduced the dosage of morphine as follows: day 1, mice received 20 mg·kg^{-1} morphine 2× a day. Day 2, mice received 30 mg·kg^{-1} morphine 2× a day. Day 3, mice received 40 mg·kg^{-1} morphine 2× day, days 4 and 5, mice received 50 mg·kg^{-1} morphine 2× a day. On the morning of day 6, mice received 50 mg·kg^{-1} morphine and approximately 2 h after the last morphine injection, mice were injected with naloxone (1 mg·kg^{-1}, s.c.) to precipitate somatic signs. This protocol allowed the detection of increased morphine withdrawal sensitivity in transgenic mice, avoiding the ceiling effect of high doses. Somatic signs were observed following naloxone injection as described above.

α3, α5 and β4 nicotinic subunit gene expression correlates with morphine withdrawal somatic signs in the BXD inbred mouse panel

Previous mouse genetic studies have phenotyped a number of morphine-induced behavioural responses across the BXD RI mouse panel (Schadt *et al.*, 2003). Analysing phenotype data along with gene expression data by means of genetic mapping and/or genetic correlations allows for the detection of putative candidate genes whose variation in expression might be responsible for the variation in behaviour observed across the BXD panel (Schadt *et al.*, 2003). We took a genetic correlation approach using publically available datasets within the GeneNetwork web resource. As described in Philip *et al.* (2010), BXD mice were given an acute i.p. injection of 50 mg·kg^{-1} morphine and 3 h later, withdrawal was precipitated with 30 mg·kg^{-1} of naloxone. While this mode of acute

morphine exposure is not similar to our repeated morphine administration protocol, it is well established that a single injection or relatively short-term chronic infusion of morphine and other drugs can elicit withdrawal-like signs ('acute dependence') in rodents. This high dose of naloxone is routinely used to induce acute precipitated morphine withdrawal in mice. The numerous similarities in the behavioural and neurobiological mechanisms mediating acute dependence and those mediating withdrawal following relatively long-term drug exposure has led to the suggestion that the effects elicited in acute dependence paradigms are valid indices of drug withdrawal and have similar predictive outcomes (Harris and Gewirtz, 2005). Withdrawal signs scored were number of jumps, faecal boli, urine puddles, locomotion, vertical activity, horizontal activity and somatic signs such as wet dog shakes, ptosis, salivation, abnormal posture and abdominal contractions. We correlated those morphine withdrawal phenotypes with basal mRNA levels of *Chrna3*, *Chrna5* and *Chrnb4* in whole brain [forebrain and midbrain dataset: INIA Brain mRNA M430 (Jun06) RMA] and many regions (hypothalamus, hippocampus, VTA, nucleus accumbens, prefrontral cortex, cerebellum, amygdala and neocortex). All comparisons used the genetic Pearson product moment correlations within GeneNetwork.

Opioid dependence human genetic analysis

Male and female European-American (n = 2772) and African-American (n = 1309) subjects from the SAGE dataset were used to assess the possible association of polymorphisms in the 15q25 gene cluster and opioid dependence. Genetic data (genotypes and phenotypes) were downloaded from dbGaP under the protocol of genetic study of nicotine dependence by X. Chen. The SAGE study is comprised of three independent studies: the Collaborative Genetic Study of Nicotine Dependence (COGEND), the Collaborative Study on the Genetics of Alcoholism (COGA) and the Family Study of Cocaine Dependence (FSCD). Genotype and phenotype data from each of the three studies were used in the analysis. European-American and African-American data were treated as two different datasets. The phenotypes tested were case/control status for opioid withdrawal and Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition (DSM-IV) opioid dependence. To conduct the analysis, single nucleotide polymorphisms (SNPs) in the 15q25 gene cluster were imputed in both datasets using IMPUTE2 (Howie *et al.*, 2009; 2011; 2012) with the 1000 Genomes Phase 1 integrated variant set (March 2012 release) as a reference panel. A total of 363 SNPs were imputed in the region containing the α3, α5 and β4 nACh receptor genes, though some markers were non-polymorphic in either dataset. Of these, 293 SNPs were shared between datasets and were used for subsequent analysis. To increase the power of our analysis, a meta-analysis was conducted using results from both datasets (n = 4081).

Statistical analysis

For all animal data, statistical analyses were performed using StatView® (SAS, Cary, NC, USA). Data were analysed with one-way ANOVA with treatment as the between subject factor or two-way ANOVA with treatment and genotype as between subject factors for the nicotinic KO and TgCHRNA5/A3/B4 mice withdrawal studies. P values of less than 0.05 were considered significant. Significant results were further analysed using the Neuman–Keuls *post hoc* test.

Statistical analysis for genetic association studies was performed using the PLINK software (Purcell *et al.*, 2007). Opioid withdrawal and DSM-IV opioid dependence were treated as dichotomized variables and were analysed using logistic regression. Age, sex, study (COGA, COGEND or FSCD), and principal components to control for population stratification within the sample were used as covariates. The GWAMA program (Magi and Morris, 2010) was used for a random effects meta-analysis of the results from both datasets. Cochrane's Q statistic P values were calculated to measure between-study heterogeneity. Correction for multiple testing was carried out on all tests jointly for the meta-analysis using single nucleotide polymorphism spectral decomposition (Nyholt, 2004), a correction for multiple testing based on the linkage disequilibrium (LD) structure of the markers tested. Based on the LD structure of the 293 markers assessed, the corrected P-value threshold was $P < 0.0006$.

Drugs

DHβE was purchased from RBI (Natick, MA, USA). The α3β4*nACh receptor-selective antagonist α-conotoxin AuIB (AuIB) was purified from the venom of the 'court cone', *Conus aulicus*, blocks the α3β4* nACh receptor subtype with >100-fold higher potency than other nicotinic receptor combinations, such as α3β2* and α4β4* (Luo *et al.*, 1998). AT-1001, a high affinity α3β4* nACh receptor antagonist (Zaveri *et al.*, 2010; Toll *et al.*, 2012) was provided by Astraea Therapeutics (Mountain View, CA, USA). Morphine sulfate and naloxone were obtained from the National Institute on Drug Abuse (Baltimore, MD, USA). AT-1001 was dissolved in 20% DMSO: emulphor : saline solution in a 1:1:18 ratio, and the drug was injected i.p. The rest of the compounds were dissolved in physiological saline (0.9% sodium chloride) and administered to each animal by s.c. or i.c.v. injection. The doses for AuIB (1.75 and 3.5 pmol) were calculated based on the functional IC_{50} at α3β4 nACh receptors (Luo *et al.*, 1998). The doses of AT-1001 were based on the recent *in vivo* studies with nicotine (Toll *et al.*, 2012).

Results

Neuronal Chrna3, Chrna4 and Chrna5 mRNA expression significantly correlates with defecation following naloxone-precipitated morphine withdrawal

As an initial screen to identify whether α3, α5 and β4 nicotinic receptor subunits might influence morphine withdrawal phenotypes, we utilized a genetic correlation analysis across the BXD inbred mouse panel using publically available datasets within the GeneNetwork web resource. We found significant correlations between mRNA levels of *Chrna3*, *Chrna5* and *Chrnb4*, in different brain regions (such as prefrontal cortex, ventral tegmental area and cerebellum), which were associated with various withdrawal signs (see Supporting Information Table S1). As an example, we illustrated, in Figure 1, the significant positive correlations between *Chrna3*, *Chrna5* and *Chrnb4* and the magnitude of defecation (number of fecal boli) in male mice during naloxone-

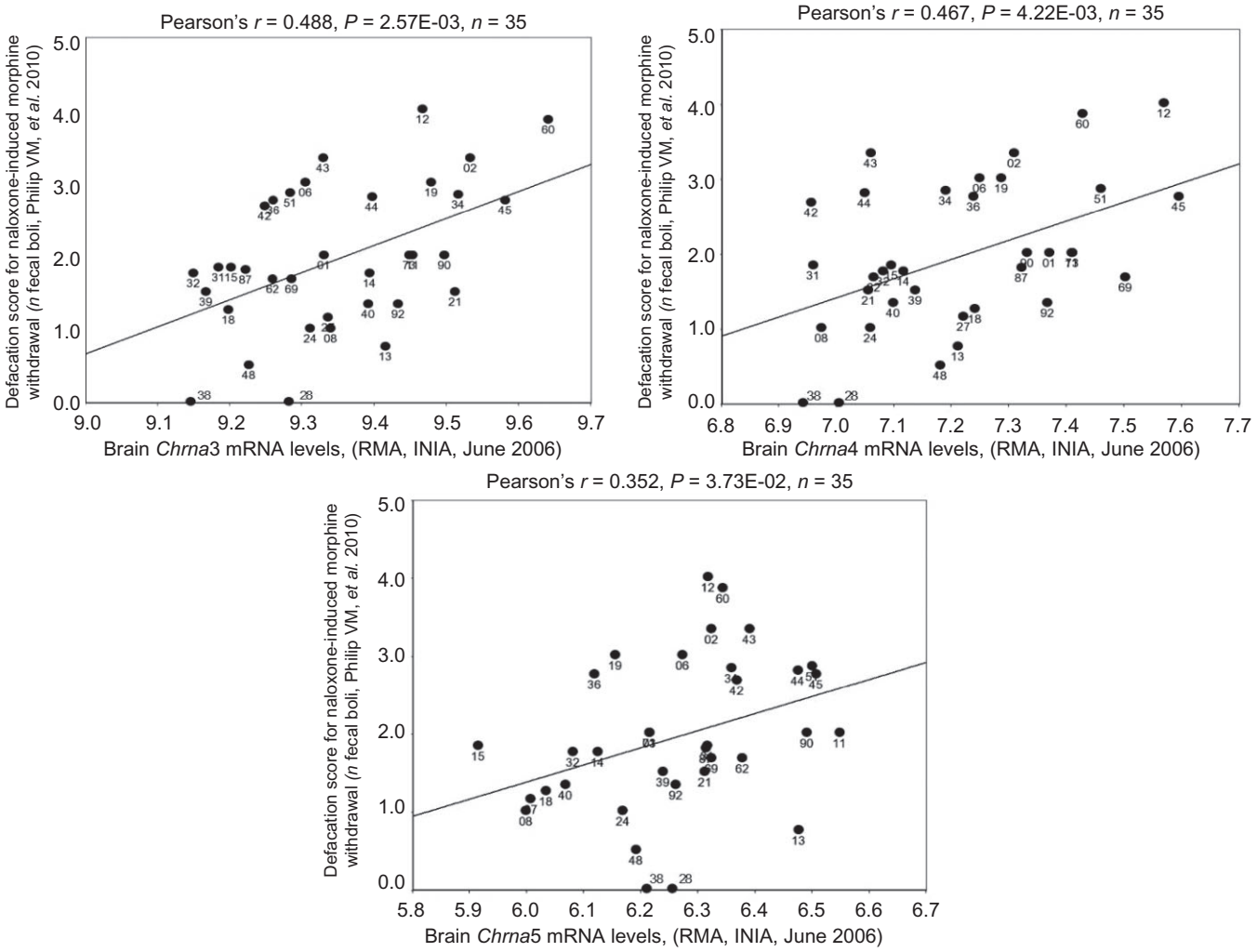

Figure 1

Correlation of α3, α5 or β4 with somatic signs across BXD strains treated with morphine. Using a publically available BXD inbred mouse panel dataset, significant positive correlations between (A) *Chrna3*, (B) *Chrna5* and (C) *Chrnb4* mRNA in the forebrain and midbrain and the magnitude of defecation in males (number of fecal boli) during naloxone-precipitated morphine withdrawal were demonstrated.

precipitated morphine withdrawal. These correlations suggest that higher mRNA expression of α3, α5, and β4 subunits within the forebrain and/or midbrain is associated with a larger withdrawal response to morphine, as measured by defecation severity. Based on these results, we considered the possibility that changes in the levels of α3, α5 and β4 subunits may affect morphine withdrawal in mice.

Morphine withdrawal modulation in transgenic TgCHRNA5/A3/B4 mice and β4 and α5 nACh receptor KO mice

Mice overexpressing the CHRNA5/A3/B4 cluster exhibited increased number of somatic signs compared with their WT littermates in morphine somatic withdrawal, resulting in a significant main effect of genotype [$F_{(1,27)}$ = 5.74, P < 0.05] and treatment [$F_{(1,27)}$ = 32.20, P < 0.0001]. Moreover, *post hoc* analysis demonstrated significant differences between TgCHRNA5/A3/B4-treated and WT-treated mice (P < 0.05; Figure 2A). The increased signs in TgCHRNA5/A3/B4-treated mice were mainly attributed to the manifestation of higher jumping behaviour, upon naloxone-precipitated withdrawal, as revealed by a significant genotype × treatment interaction [$F_{(1,27)}$ = 6.21, P < 0.05], followed by a *post hoc* test (Table 1). Furthermore, a higher percentage of TgCHRNA5/A3/B4 mice presented diarrhoea compared with their WT counterparts (Table 1).

Conversely, the increase in total somatic signs observed in β4 nACh receptor WT mice was significantly reduced in β4 nACh receptor KO mice, revealing a significant main effect of treatment [$F_{(1,22)}$ = 181.9, P < 0.0001] and genotype [$F_{(1,22)}$ = 19.77, P < 0.001] (Figure 2B). This was due to a decrease in head shakes resulting in a genotype × treatment interaction [$F_{(1,22)}$ = 30.00, P < 0.0001] and body tremors resulting in a genotype × treatment interaction [$F_{(1,22)}$ = 12.48, P < 0.001] upon naloxone-precipitated withdrawal, but not jumps in the β4 nACh receptor KO mice compared with their WT counterparts (Table 2). In contrast, all individual signs were

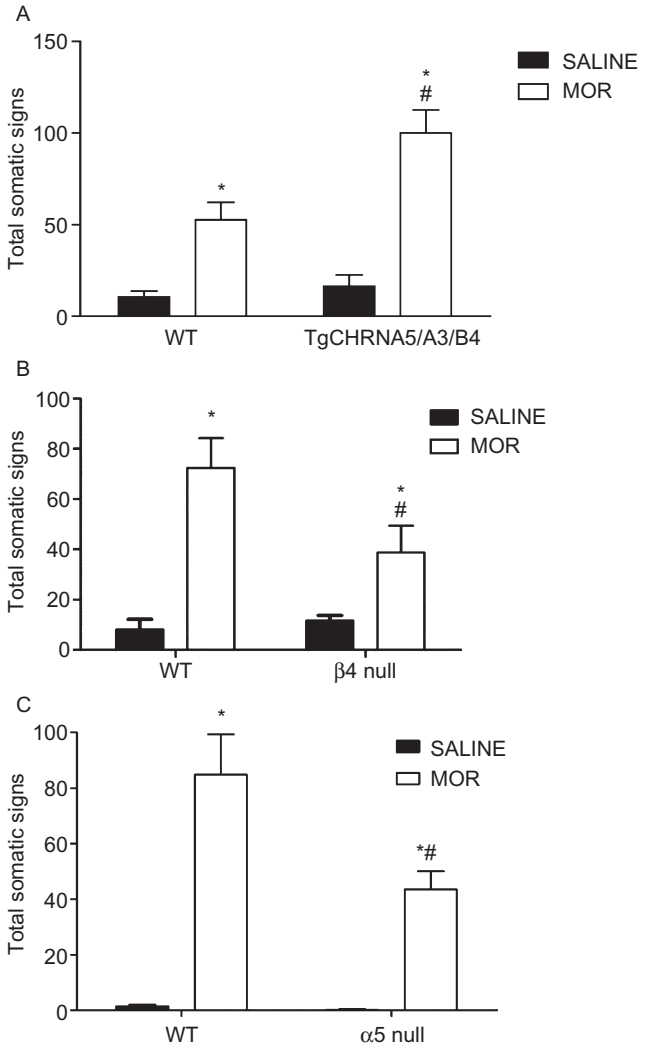

Figure 2

Overexpression of the genomic cluster CHRNA5/A3/B4 in mice increases somatic signs associated with morphine withdrawal. TgCHRNA5/A3/B4 morphine-dependent mice demonstrate a significant increase in withdrawal somatic signs compared with their WT counterparts. Data are expressed as mean ± SEM of $n = 6$–9 mice per group. *$P < 0.05$ versus saline control; #$P < 0.05$ versus WT-treated mice. (B) Conversely, β4 nACh receptor KO morphine-dependent mice expressed significant decreases in naloxone-precipitated somatic withdrawal signs compared with their WT counterparts. Data are expressed as mean ± SEM. of $n = 6$–8 mice per group. *$P < 0.05$ versus saline control; #$P < 0.05$ versus WT-treated mice. (C) Furthermore, α5 nACh receptor KO morphine-dependent mice demonstrated a significant decrease in withdrawal somatic signs compared with their WT counterparts. Data are expressed as mean ± SEM. of $n = 6$–8 mice per group. *$P < 0.05$ versus saline control; #$P < 0.05$ versus WT-treated mice. MOR, morphine.

attenuated in morphine-dependent α5 nACh receptor KO mice (Table 3). Indeed, the increase in total somatic signs observed in α5 nACh receptor WT mice was significantly reduced in the α5 KO group (Figure 2C), demonstrating a significant main effect of treatment [$F_{(1,18)} = 41.47$, $P < 0.001$] and genotype [$F_{(1,18)} = 4.67$, $P < 0.05$] in α5 nACh receptor KO

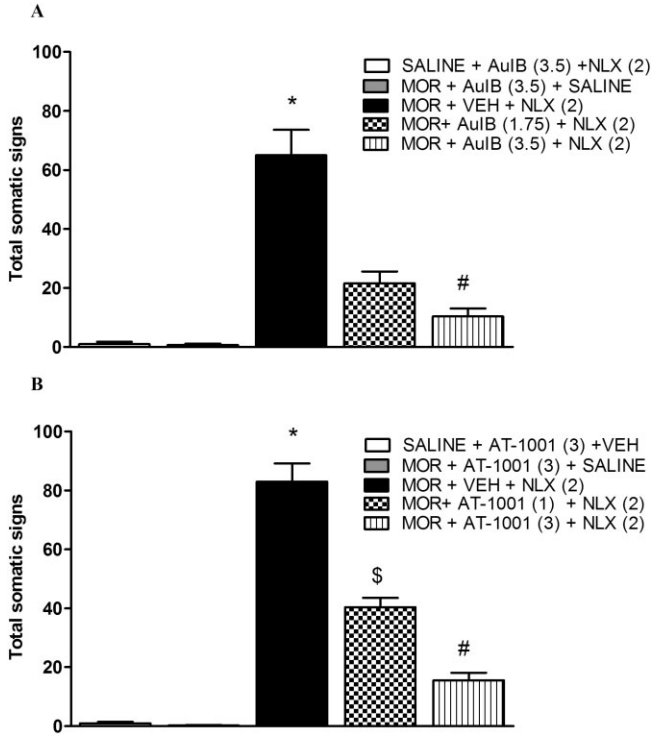

Figure 3

AuIB and AT-1001 reduce physical morphine withdrawal signs in C57Bl/6 mice. Morphine-dependent mice pretreated with (A) 1.75 or 3.5 pmol AuIB displayed a decrease in somatic withdrawal signs. *$P < 0.05$ versus all treatment groups; #$P < 0.05$ versus morphine (MOR) + AuIB (1.75 pmol) + naloxone (NLX) (2) group. (B) Similarly, 1 and 3 mg·kg^{-1} AT-1001 reduced the expression of somatic withdrawal signs. *$P < 0.05$ versus all treatment groups; #$P < 0.05$ versus MOR+ AT-1001 (1) + NLX (2) group. The total withdrawal signs measure consists of paw tremors, head shakes, backing, ptsosis, diarrhoea, jumping and other miscellaneous signs. Data are expressed as mean ± SEM. of $n = 6$–8 mice per group. VEH, vehicle.

and WT. In addition, two-way ANOVA revealed the difference was due to the decrease in signs of paw tremors [$F_{(1,18)} = 10.77$, $P < 0.004$], head shakes [$F_{(1,18)} = 14.94$, $P < 0.001$] and jumping [$F_{(1,18)} = 60.43$, $P < 0.0001$].

α3β4 nACh receptor antagonists block physical signs of morphine withdrawal*

We further studied the role of α3β4* nACh receptor inhibition in morphine withdrawal by using two selective α3β4* antagonists in morphine-dependent mice. Male mice chronically treated with saline or morphine received i.c.v. injections of either vehicle or the α3β4* antagonist, AuIB (1.75 or 3.5 pmol per mouse) before naloxone (2 mg·kg^{-1}, s.c.) challenge. As expected, naloxone precipitated a significant increase in total somatic signs in morphine-treated vehicle-injected mice [$F_{(4,18)} = 3.35$, $P < 0.05$] while AuIB significantly reduced naloxone-precipitated somatic signs [$F_{(4,28)} = 3.058$, $P < 0.05$)] (Figure 3A). All individual signs were attenuated by AuIB treatment (Table 4). Similarly, AT-1001, another selective α3β4* antagonist active after systemic administration, given at 1 and 3 mg·kg^{-1} blocked naloxone-precipitated

Table 1

Somatic signs of morphine withdrawal in mice overexpressing the genomic cluster CHRNA5/A3/B4

Individual somatic signs	WT SAL	TgCHRNA5/A3/B4 SAL	WT MOR	TgCHRNA5/A3/B4 MOR
Paw tremors	2.4 ± 0.9	7.3 ± 4.6	13.8 ± 4.9	25.4 ± 6.9
Head shakes	0.4 ± 0.4	1.4 ± 0.5	1.7 ± 0.4	2.5 ± 0.4
Backing	0 ± 0	0 ± 0	8.6 ± 2.9	4.4 ± 1.7
Ptosis	0.6 ± 0.6	0 ± 0	4.8 ± 1.0	4.0 ± 0.9
Jumping	0 ± 0	0.3 ± 0.2	8.3 ± 4.5*	42.8 ± 9.0*#
Body tremor	0.9 ± 0.9	0.6 ± 0.4	2.3 ± 1.0	3.8 ± 0.8
Occurrence of diarrhoea (% of mice)	0	0	33	88

Mice were made dependent on morphine for 5 days. On day 6, mice were administered naloxone (1 mg·kg^{-1}, s.c.) before observing individual physical signs and % occurrence of diarrhoea. Data are expressed as mean ± SEM of n = 6–9 mice per group. *Denotes $P < 0.05$ versus saline control.
#$P < 0.05$ versus WT-treated mice.
MOR, morphine.

Table 2

Knockout of β4 gene reduced somatic signs in morphine-treated mice

Individual somatic signs	β4 WT SAL	β4 KO SAL	β4 WT MOR	β4 KO MOR
Paw tremors	4.5 ± 0.8	7.2 ± 0.5	22.8 ± 5.1*	11.9 ± 2.5#
Head shakes	2.8 ± 0.8	4.3 ± 0.5	25.5 ± 6.1*	8.9 ± 2.0#
Backing	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Ptosis	0 ± 0	0 ± 0	2.6 ± 0.7	1.3 ± 0.7
Jumping	0.7 ± 0.3	0 ± 0	15.6 ± 7.2*	14 ± 5.8*
Body tremor	0 ± 0	0 ± 0	6.9 ± 1.0*	2.7 ± 0.6*#
Occurrence of diarrhoea (% of mice)	0	0	50	0

Mice were made dependent on morphine for 8 days. On day 9, mice were administered naloxone (2 mg·kg^{-1}) before observing individual physical signs expressed as mean ± SEM and % occurrence of diarrhoea.
n = 8 mice per group.
*$P < 0.05$ from saline group.
#$P < 0.05$ from morphine WT group.
MOR, morphine; SAL, saline.

somatic signs [$F_{(4,27)}$ = 112.361, $P < 0.0001$] in morphine-dependent mice (Figure 3B). The highest doses of AuIB (3.5 pmol) and AT-1001 (3 mg·kg^{-1}) had no significant effect in saline-naloxone or morphine-saline-treated mice.

Role of the α4β2 nACh receptor subtype in morphine physical dependence*

As a control, we examined the role of α4β2* nACh receptor inhibition in morphine withdrawal. Male mice chronically treated with saline or morphine were treated with vehicle or the β2* selective nACh receptor antagonist DHβE (3 mg·kg^{-1}, s.c.) 5 min before naloxone (2 mg·kg^{-1}, s.c.) challenge. Mice treated with morphine showed significant naloxone-precipitated somatic signs [$F_{(3,20)}$ = 25.79, $P < 0.0001$]. DHβE failed to significantly alter the intensity of morphine withdrawal (Figure 4A). DHβE at the dose tested (3 mg·kg^{-1}, s.c.) had no significant effect in saline-naloxone or morphine-saline-treated mice. Furthermore, morphine withdrawal signs were not significantly altered in the α4 (Figure 4B) [$F_{(1,22)}$ = 0.24, $P > 0.5$] or the β2 (Figure 4C) [$F_{(1,31)}$ = 2.47, $P > 0.1$] nACh receptor KO mice compared with their respective WT controls. There was no significant increase in control, saline-treated, WT or KO mice challenged with naloxone. In addition, there was no significant difference in individual somatic signs between genotypes.

Variants in the 15q25 gene cluster are associated with opioid withdrawal and DSM-IV opioid dependence

Prompted by the results from animal studies, we performed association analyses using the SAGE European-American and African-American datasets to determine if variants in the 15q25 gene cluster are associated with opioid dependence based on case/control status of opioid withdrawal and

Table 3

Knockout of α5 gene reduced somatic signs in morphine-treated mice

Individual somatic signs	α5 WT SAL	α5 KO SAL	α5 WT MOR	α5 KO MOR
Paw tremors	1.2 ± 0.4	0.3 ± 0.2	33 ± 13.2*	12.5 ± 3.0#
Head shakes	0.3 ± 0.2	0 ± 0	1.8 ± 0.7*	2.3 ± 0.7*#
Backing	0 ± 0	0 ± 0	2.5 ± 0.9	1.2 ± 0.3
Ptosis	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Jumping	0 ± 0	0.2 ± 0.2	44.5 ± 7.7*	26.0 ± 4.88*#
Body tremor	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Occurrence of diarrhoea (% of mice)	0	0	100	50

Mice were made dependent on morphine for 8 days. On day 9, mice were administered naloxone (2 mg·kg^{-1}) before observing individual physical signs expressed as mean ± SEM and % occurrence of diarrhoea.
*Denotes difference from vehicle group. n = 8 mice per group.
*P < 0.05 from control group. #P < 0.05 from Morphine WT group.
MOR, morphine; SAL, saline.

Table 4

AuIB treatment dose-dependently attenuated all individual signs in morphine somatic withdrawal

Individual somatic signs	SAL + AuIB (3.5) + NLX (2)	MOR + AuIB (3.5) + SAL	MOR + SAL + NLX (2)	MOR + AuIB (1.75) + NLX (2)	MOR + AuIB (3.5) + NLX (2)
Paw tremors	0 ± 0	0 ± 0	40.4 ± 5.7*	12.0 ± 2.0#	3.0 ± 1.1#
Head shakes	1 ± 1.0	1.0 ± 0.5	4.1 ± 1.0	1.1 ± 0.5#	1.6 ± 1.0#
Backing	0 ± 0	0 ± 0	1.1 ± 0.4	0 ± 0	0 ± 0
Ptosis	0 ± 0	0 ± 0	1.0 ± 0.3	0.3 ± 0.3	1.0 ± 1.0
Writhing	0 ± 0	0 ± 0	0 ± 0	0.1 ± 0.1	0.4 ± 0.4
Jumping	0 ± 0	0 ± 0	18.0 ± 3.0*	7.7 ± 3.0#	4.2 ± 3#
Body tremor	0 ± 0	0 ± 0	1.0 ± 0.3	0.4 ± 0.2	0.4 ± 0.2
Occurrence of diarrhoea (% of mice)	0	0	100	43	14

Mice were made dependent on morphine for 8 days. On day 9, mice were administered naloxone (2 mg·kg^{-1}) before observing individual physical signs expressed as mean ± SEM and % occurrence of diarrhoea.
n = 6 mice per group.
*P < 0.05 from control group. Dose of AuIB is shown in pmol.
#P < 0.05 from morphine + VEH + NLX (2) group.
MOR, morphine; SAL, saline; NLX, naloxone.

DSM-IV opioid dependence. Results shown in Table 5 represent the top three most significant associations detected of the 293 SNPs analysed. The variants rs112712252 and rs190825809, both located in introns in the CHRNA3 gene, were significantly associated with a protective effect against opioid withdrawal and DSM-IV opioid dependence in both European and African-American populations. The variant rs116932868, also located in an intron in CHRNA3, was also associated with a protective effect for both phenotypes measured in European-Americans, but only for DSM-IV opioid dependence in African-Americans, though the significance in opioid withdrawal was marginal (P = 0.06). Meta-analysis also revealed a significant protective effect for these three variants in both phenotypes. All three variants survived correction for multiple testing for both phenotypes.

Although the α5 SNP rs16969968 was not found to be among the top three most significant associations in this study, we also report the results of this variant in Table 5 due to its significant role in nicotine dependence, and to support previous findings showing an association with this variant in opioid dependence (Erlich *et al.*, 2010; Sherva *et al.*, 2010). The rs16969968 marker was significantly associated with risk for DSM-IV opioid dependence in the European-American population, and in the meta-analysis, though this P-value did not survive correction for multiple testing. The variant was not associated with opioid withdrawal in this study.

A LD block of the top three most significant SNPs and rs16969968 is shown in Figure 5. While rs16969968 is not in LD with the other three SNPs, rs112712252, rs190825809 and rs116932868 are in strong LD in the European-American sample (Figure 5A), African-American sample (Figure 5B) and the combined sample (Figure 5C), suggesting that these SNPs are strongly correlated to opioid dependence.

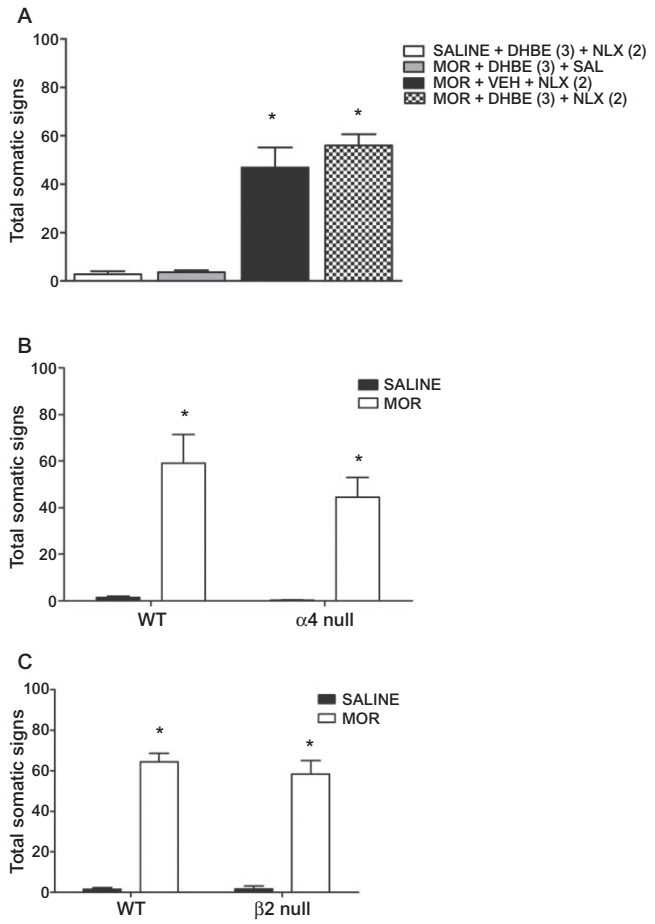

Figure 4

Role of the α4β2* subtype in morphine withdrawal somatic signs. Morphine-dependent C57Bl/6 mice treated with (A) vehicle or DHβE (2 mg·kg^{-1}, s.c.) and challenged with naloxone (NLX) did not alter somatic withdrawal signs nor did (B) β2nACh receptor KO or (C) α4 nACh receptor KO compared with their respective WT littermates. *P < 0.05 versus saline and drug control. MOR, morphine; VEH, vehicle.

Discussion

The goal of the present study was to elucidate the role of α3β4* nACh receptors in a mouse model of physical morphine withdrawal using multiple approaches: mouse genetics, preclinical pharmacology and human genetic association studies. Our BXD RI mouse panel data demonstrated that higher mRNA expression of α3, α5 and β4 nACh receptor subunits within the forebrain and/or midbrain is associated with higher intensity of one of the morphine withdrawal signs. This notion that α5, α3 and β4 nACh receptor subunits are involved in morphine dependence is confirmed in mice bearing extra copies of the cluster CHRNA5/A3/B4, thus mice with increased expression of α5, α3 and β4 nACh receptor subunits exhibit enhanced somatic signs of withdrawal. In agreement with this, mouse genetic KO and pharmacological studies demonstrated that the blockade of α3β4* nACh receptors reduced the expression and development of the physical signs associated with morphine withdrawal. Furthermore, the α5 nACh receptor subunit, which can co-assemble with the α3β4* nACh receptor subtype, seems to be partially mediating the somatic signs of morphine withdrawal. Lastly, association analyses in two human datasets showed that variants of the CHRNA3 gene are associated with opioid dependence and withdrawal.

Our genetic correlation analyses of morphine withdrawal phenotype data and gene expression data across the BXD RI panel demonstrate that mRNA levels of α3, α5 and β4 nACh receptor subunits in many brain regions are associated with higher magnitude of morphine somatic signs. In particular, these subunits within the forebrain, midbrain and other brain regions are associated with higher magnitude of defecation in male mice during naloxone-precipitated morphine withdrawal. Overall, the BXD RI data suggest that higher mRNA expression of α3, α5 and β4 subunits in the brain is associated with a larger withdrawal response to morphine. Moreover, mice overexpressing the cluster CHRNA5/A3/B4 with increased α3, α5 and β4 expression demonstrated enhanced morphine physical withdrawal compared with their WT counterparts.

Indeed, the reduction in morphine withdrawal signs in the α5 nACh receptor KO mice and the increase in somatic signs in the TgCHRNA5/A3/B4 mouse model suggest that the α3α5β4* nACh receptor subtype is involved in the somatic symptoms of morphine abstinence in the mouse. Interestingly, the α5 nACh receptor subunit co-assembles with α3β4* nACh receptor subtypes to form functional receptors in the periphery (ganglia) and CNS (MHb and IPN; Wada *et al.*, 1990; Zoli *et al.*, 1995; Quick *et al.*, 1999; Whiteaker *et al.*, 2002). In addition, TgCHRNA5/A3/B4 exhibit increased activation of the medial habenula upon nicotine administration (Gallego *et al.*, 2012). Our current data does not allow us to discern whether α3β4* nACh receptors that incorporate the α5 subunit are responsible for the morphine withdrawal increase detected in TgCHRNA5/A3/B4. In nicotine somatic withdrawal signs, neuronal α3β4* nACh receptor subtypes mediate somatic signs of withdrawal independently of α5 nACh receptor subunits (Jackson *et al.*, 2013).

Conversely, the α3β4* selective antagonist, AuIB, given centrally, blocked the expression of morphine physical signs in a dose-related manner. We believe that the doses used (1.75 or 3.5 pmol per mouse) in our current study selectively blocked the α3β4* nACh receptor subtype. Our highest dose of 3.5 pmol would yield, based on diffusion studies from an estimated tissue concentration of ≈ 0.7 μM, a value similar to the IC_{50} value (0.75 μM) obtained with rat α3β4 nACh receptors expressed in Xenopus oocytes (Luo *et al.*, 1998) and the IC_{50} value (2.2 μM) that inhibits nicotine-induced hippocampal noradrenaline secretion (Fu *et al.*, 1999). AuIB is 100-fold more potent at α3β4* nACh receptors compared with other heteromeric nicotinic receptor combinations and 10-fold more potent at α3β4* than at the α7 homomeric nACh receptor subtype (Luo *et al.*, 1998). The role of α3β4* nACh receptors in morphine withdrawal is further supported by the experiments with another selective antagonist, AT-1001 (Toll *et al.*, 2012). The pharmacological results were supported by the data obtained in β4 nACh receptor KO mice, demonstrating a significant reduction of total somatic signs compared with their WT littermates, and by the correlation results with brain α3, α5 and β4 nACh receptor subunit mRNA levels in

Table 5

Variants in the 15q25 gene cluster are associated with opioid dependence

			SAGE EA, *n* = 2772		SAGE AA, *n* = 1309		Combined, *n* = 4081		
Phenotype	**SNP**	**Allele**	**OR**	***P***	**OR**	***P***	**OR**	***P***	**Q_*P***
Withdrawal	rs112712252	G	0.31	**7.3E–04**	0.31	**0.01**	0.31	**3.1E–05***	0.99
	rs190825809	C	0.33	**0.001**	0.32	**0.02**	0.33	**9.6E–05***	0.98
	rs116932868	G	0.31	**0.001**	0.33	0.06	0.32	**2.0E–04***	0.92
	rs16969968	A	1.15	0.22	1.49	0.32	1.17	0.14	0.54
DSM-IV	rs112712252	G	0.29	**1.8E–04**	0.27	**0.006**	0.28	**3.5E–06***	0.91
	rs190825809	C	0.28	**3.0E–04**	0.28	**0.01**	0.29	**1.1E–05***	0.91
	rs116932868	G	0.30	**3.3E–04**	0.28	**0.03**	0.28	**2.4E–05***	0.99
	rs16969968	A	1.25	**0.04**	1.68	0.22	1.28	**0.02**	0.50

Association analysis conducted in the SAGE European American (EA) and African-American (AA) datasets shows that variants in the 15q25 gene cluster are significantly associated with a protective effect against opioid dependence. Uncorrected *P* values are shown for individual datasets. Cochran's Q statistic *P* values (Q_*P*) show no significant heterogeneity between datasets. Significant results are bold and underlined. *Denotes results that survived the single nucleotide polymorphism spectral decomposition correction for multiple testing threshold ($P < 0.0006$).

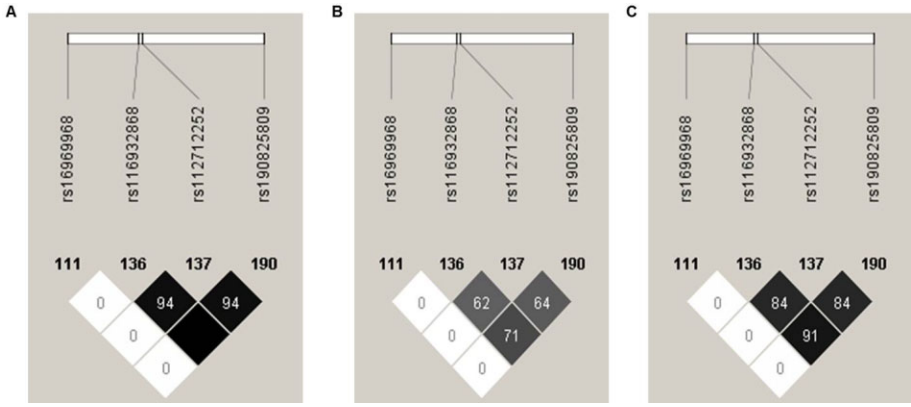

Figure 5

Linkage disequilibrium (LD) blocks containing r^2 values for (A) the SAGE European-American dataset, (B) the SAGE African-American dataset and (C) both SAGE datasets combined. The darker the shading and/or higher numbers represent a stronger correlation between two markers.

the BXD RI mice. Our findings complement previous findings with non-selective nicotinic antagonists, mecamylamine and BTMPS (Hall *et al.*, 2011; Taraschenko *et al.*, 2005), and with 18-MC, a moderately selective α3β4* nACh receptor antagonist, which have been shown previously to reduce morphine physical dependence signs in rats (Taraschenko *et al.*, 2005). The importance of the α3β4* nACh receptor subtype in morphine physical dependence is highlighted by the observation that α4β2* nACh receptors, the major heteromeric subtype expressed in the CNS, do not participate in morphine somatic withdrawal signs. Neither the selective β2* nACh receptor antagonist, DHβE, nor the β2 and α4 nACh receptor KO mice demonstrated significant alterations in somatic signs of withdrawal.

While the brain regions that mediate the blocking effect of α3β4* antagonists on morphine physical dependence were not investigated in our experiments, the limited brain distribution of α3β4* nACh receptors suggests the MHb-IPN pathway as a possible site. In line with this suggestion, local administration of the α3β4* nACh receptor antagonist, 18-MC into the MHb and IPN significantly reduces somatic morphine withdrawal signs in rats (Panchal *et al.*, 2005). However, it is possible that other brain regions such as the ventral tegmental area, hippocampus and cortex, where α3, α5 and β4 nACh receptor subunits are also found, may be involved in morphine withdrawal (Millar and Gotti, 2009). Interestingly, Neugebauer *et al.* (2013) recently reported that knocking down the β4 subunits in the MHb did not reduce the number of jumps during somatic morphine withdrawal. These findings are similar to our data with the β4 KO mice, which did not differ in the number of jumps compared with their WT counterparts. In addition, our findings with BXD mice gene correlations suggest this possibility. Interestingly, our pharmacological and genetic approaches indicate that

the α3β4* nACh receptor subtype mostly mediates naloxone-precipitated jumping behaviour and diarrhoea somatic signs. These two manifestations of morphine physical dependence are principally mediated by the locus coeruleus (LC; Maldonado *et al.*, 1992), a hindbrain region where α3β4* nACh receptors are also expressed (Lena *et al.*, 1999). Therefore, we cannot exclude their possible involvement in morphine dependence since direct infusion of 18-MC into the LC is able to attenuate most of the somatic signs associated with withdrawal (Panchal *et al.*, 2005).

Polymorphisms in the CHRNA5/CHRNA3/CHRNB4 gene cluster were recently associated with increased risk and severity of opioid dependence (Erlich *et al.*, 2010; Sherva *et al.*, 2010), further supporting a role for nACh receptors in mediating aspects of morphine withdrawal. In agreement, the results of our human genetic association study implicate a protective role for the rs112712252, rs190825809 and rs116932868 variants, located within the CHRNA3 gene in opioid withdrawal and DSM-IV criteria. These results support findings from the current animal study, where α3β4* nACh receptor antagonists significantly reduced physical morphine withdrawal signs in the mouse. To our knowledge, this is the first known study to identify significant variation in the CHRNA3 gene implicated in opioid dependence phenotypes. Because the markers are in LD (i.e. the markers are strongly correlated), it is difficult to identify the causal variant in this case and we cannot rule out the possibility that these markers serve as proxies for a causal variant that was not identified in this study. Nonetheless, our findings identify protective variants in the CHRNA3 gene in opioid dependence and withdrawal. Our assessment of the rs16969968 variant, located in CHRNA5, did not survive correction for multiple testing, but for the DSM-IV phenotype in the combined sample, produced a similar odds ratio and *P* value similar to that observed in the Sherva *et al*. (2010) study, which also used DSM-IV opioid dependence as a phenotypic measure. In both studies, rs16969968 was associated with risk for opioid dependence. Overall, the human results identify alleles with reduced risk for opioid dependence in the 15q25 gene cluster. Future studies should involve haplotype analyses across the genetic region to identify the most significant marker combinations that contribute to opioid dependence phenotypes.

In summary, our findings suggest that neuronal α3β4* nACh receptors are a potential target for treating physical morphine dependence, and may be involved in mechanisms of physical drug withdrawal in general.

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Authors' contributions

Participated in research design: Muldoon, Perez, Maldonado, Dierssen, De Biasi, Jackson, Chen and Damaj. Conducted experiments: Muldoon, Jackson, Perez, Molas, Harenza, Anwar and Rais. Contributed new reagents or analytic tools: McIntosh and Zaveri. Performed data analysis: Muldoon, Perez, Molas, Jackson, Chen, De Biasi and Damaj. Wrote or contributed to the writing of the manuscript: Muldoon, Jackson, Perez, Molas, Harenza, Rais, Anwar, Zaveri, Dierssen, Maskos, McIntosh, Miles, Chen, De Biasi and Damaj.

Conflict of interest

All of the authors declare no conflict of interest.

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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:

http://dx.doi.org/10.1111/bph.12741

Table S1 Correlation of α3, α5 or β4 with somatic signs across BXD strains treated with morphine in various brain regions. Using a publically available BXD inbred mouse panel dataset (Philip *et al*., 2010), we assessed significant correlations between Chrna3, Chrna5 and Chrnb4 mRNA levels in several brain regions and the scores of various morphine withdrawal signs. Locomotion is defined by the number beam breaks. Horizontal is defined by the horizontal distance traveled in the open field test.