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## **Alterations in nicotinic receptor alpha5 subunit gene differentially impact early and later stages of cocaine addiction: a translational study in transgenic rats and patients**

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1 **Alterations in nicotinic receptor alpha5 subunit gene differentially impact early and later**  
2 **stages of cocaine addiction: a translational study in transgenic rats and patients**  
3

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## Abstract

Cocaine addiction is a chronic and relapsing disorder with an important genetic component. Human candidate gene association studies showed that the single nucleotide polymorphism (SNP) rs16969968 in the  $\alpha 5$  subunit ( $\alpha 5$ SNP) of nicotinic acetylcholine receptors (nAChRs), previously associated with increased tobacco dependence, was linked to a lower prevalence of cocaine use disorder (CUD). Three additional SNPs in the  $\alpha 5$  subunit, previously shown to modify  $\alpha 5$  mRNA levels, were also associated with CUD, suggesting an important role of the subunit in this pathology. To investigate the link between this subunit and CUD, we submitted rats knockout for the  $\alpha 5$  subunit gene ( $\alpha 5$ KO), or carrying the  $\alpha 5$ SNP, to cocaine self-administration (SA) and showed that the acquisition of cocaine-SA was impaired in  $\alpha 5$ SNP rats while  $\alpha 5$ KO rats exhibited enhanced cocaine-induced relapse associated with altered neuronal activity in the nucleus accumbens. In addition, we observed in a human cohort of patients with CUD that the  $\alpha 5$ SNP was associated with a slower transition from first cocaine use to CUD. We also identified a novel SNP in the  $\beta 4$  nAChR subunit, part of the same gene cluster in the human genome and potentially altering *CHRNA5* expression, associated with shorter time to relapse to cocaine use in patients.

In conclusion, the  $\alpha 5$ SNP is protective against CUD by influencing early stages of cocaine exposure while *CHRNA5* expression levels may represent a biomarker for the risk to relapse to cocaine use. Drugs modulating  $\alpha 5$  containing nAChR activity may thus represent a novel therapeutic strategy against CUD.

**Keywords:** cocaine use disorder, transgenic rats, nicotinic receptors, human polymorphisms, relapse, nucleus accumbens.

60

## 61 **1.1 Introduction**

62

63 Cocaine use disorder (CUD) is a clinically devastating neuropsychiatric disease without any  
64 approved pharmacotherapy (Degenhardt *et al.*, 2011). Relapse to cocaine seeking after a phase of  
65 abstinence is the main impediment in the treatment of CUD, with 78% of patients relapsing  
66 within three months after discharge from hospital detoxification (Lima *et al.*, 2019). In such  
67 pathological cocaine users, relapse can be precipitated by exposure to cocaine-associated cues,  
68 stress, or to cocaine itself (Childress *et al.*, 1993; Jaffe *et al.*, 1989; O'Brien, 1998; Sinha, 2001).  
69 Relapse prevention is thus a key target for clinicians in the treatment of substance use disorders  
70 in general since it has been associated with the chronicization and the burden of these disorders  
71 (Hendershot *et al.*, 2011). Similarly, reinstatement of cocaine-seeking can be elicited in  
72 laboratory animals after extinction of cocaine self-administration (SA), by exposure to a  
73 conditioned stimulus (CS) previously associated with cocaine intake, stress or a cocaine-priming  
74 injection (Crombag and Shaham, 2002; de Wit and Stewart, 1981; Epstein *et al.*, 2006; Fuchs *et*  
75 *al.*, 1998).

76 Cocaine acts primarily by inhibiting monoamine transporters, such as the dopamine transporter  
77 (DAT), leading to increased extracellular dopamine concentration in the nucleus accumbens  
78 (NAcc), an effect considered essential for its reinforcing property. However, cocaine is also an  
79 inhibitor of nicotinic acetylcholine receptors (nAChRs) (Damaj *et al.*, 1999; Francis *et al.*, 2000),  
80 an action that can alter dopamine release in the NAcc (Acevedo-Rodriguez *et al.*, 2014). nAChRs  
81 are pentameric ligand-gated ion channels composed of  $\alpha$  ( $\alpha 2$ –  $\alpha 7$ ,  $\alpha 9$ ,  $\alpha 10$ ) and  $\beta$  ( $\beta 2$ –  $\beta 4$ )  
82 subunits that co-assemble according to various combinations with distinct brain localizations and

83 functional properties (Changeux, 2010). nAChRs have been investigated in preclinical models of  
84 cocaine addiction. For example, the nAChR antagonist mecamylamine reduces cocaine-SA and  
85 prevents cocaine-SA escalation in rodents (Blokhina *et al.*, 2005; Hansen and Mark, 2007; Levin  
86 *et al.*, 2000). Additionally, 18-MC, an antagonist of the  $\alpha 3\beta 4$  containing (\*) nAChRs, decreases  
87 cocaine-SA in rats (Glick *et al.*, 1996; Pace *et al.*, 2004), and mice knockout (KO) for the  $\beta 2$   
88 nAChR subunit exhibit decreased cocaine-induced conditioned place preference (Zachariou *et*  
89 *al.*, 2001). Interestingly, mecamylamine also reduces craving for cocaine in CUD subjects (Reid  
90 *et al.*, 1999).

91 Several independent large-scale human genome-wide association studies (GWAS) identified  
92 variants in a region on human chromosome 15 encoding the  $\alpha 3$ ,  $\alpha 5$  and  $\beta 4$  nAChR subunits that  
93 increase the risk for nicotine dependence (Bierut *et al.*, 2007; Bierut *et al.*, 2008; Sherva *et al.*,  
94 2008). One of these variants, the non-synonymous coding SNP rs16969968 found in exon 5 of  
95 the  $\alpha 5$  gene ( $\alpha 5$ SNP) that causes an amino acid change D398N in the second intracellular loop of  
96 the  $\alpha 5$  subunit, has been recently introduced into the genome of the rat, resulting in increased  
97 nicotine SA at high doses and relapse (Forget *et al.*, 2018). This  $\alpha 5$ SNP is very frequent in the  
98 general population and may induce a partial loss of function of the corresponding pentamer  
99 (Bierut *et al.*, 2008; Frahm *et al.*, 2011; Kuryatov *et al.*, 2011) without altering the overall  
100 expression of the subunit, at least in rats (Forget *et al.*, 2018). It does not modify the  
101 electrophysiological function of the receptor when expressed in concatemer in *Xenopus* oocytes  
102 (Prevost *et al.*, 2020), and it lies away from agonists' binding site, suggesting that it affects other  
103 features of the receptor, but its precise mechanism of action is currently unknown (Maskos,  
104 2020). Interestingly, in candidate gene association studies, the  $\alpha 5$ SNP has also been associated  
105 with a lower prevalence of CUD in humans (Grucza *et al.*, 2008; Sherva *et al.*, 2010). In addition,

106 three other SNPs in the  $\alpha 5$  nAChR subunit, linked to altered levels of  $\alpha 5$  mRNA in humans, have  
107 also been associated with CUD (Sherva *et al.*, 2010; Wang *et al.*, 2009). These genetic studies  
108 suggest an involvement of the  $\alpha 5$  nAChR subunit in cocaine dependence, although the precise  
109 mechanisms and stages of CUD implicated have never been further assessed so far.

110 In fact, multiple behaviors related to CUD may be impacted by alterations in  $\alpha 5$ \*nAChR density  
111 or mutations in the  $\alpha 5$  nAChR subunit gene, that now need to be identified through longitudinal  
112 studies to understand how they contribute to the risk for this pathology. For this, the first goal of  
113 the present study was to clarify the role of  $\alpha 5$ \*nAChRs and the impact of the  $\alpha 5$ SNP, found to  
114 confer protection for CUD in humans, on the different stages of an intravenous (IV) cocaine-SA  
115 procedure, from first cocaine intake to relapse to cocaine seeking after SA extinction, using  
116 transgenic rat lines previously generated in the laboratory (Forget *et al.*, 2018). At the molecular  
117 level, since cocaine has been shown to inhibit nAChR-mediated currents in midbrain DA neurons  
118 (Acevedo-Rodriguez *et al.*, 2014), we hypothesized that the association between  $\alpha 5$ \*nAChRs and  
119 CUD may be linked to an implication of the  $\alpha 5$  subunit in nAChR response to cocaine. Thus, the  
120 second objective of the study was to characterize the influence of the  $\alpha 5$  subunit on nAChR  
121 functional response to cocaine *in vitro*. Finally, for translational purposes, we took advantage of  
122 the information generated by the SA longitudinal study implemented in our preclinical models to  
123 i) clarify, in humans, the association of the  $\alpha 5$ SNP with specific CUD phenotypes, and ii) seek  
124 for novel polymorphisms in the *CHRNA5/A3/B4* gene cluster, linked to  $\alpha 5$  expression levels, in  
125 association with these CUD phenotypes.

## 126 **1.2 Material and Methods**

127

128 **1.2.1 Cocaine-SA in transgenic rats**

129

130 **1.2.1.1 Experimental Model and Subject Details**

131

132 Male rats experimentally naive at the start of the study and initially weighing 250 to 275 g were  
133 used for all behavioral experiments. All rats were individually housed in a temperature-controlled  
134 environment on a 12-h reverse light/dark cycle (lights off from 08:00 hours to 20:00 hours) at the  
135 start of the experiments and were mildly food restricted (20g/day/rat).

136 Wild-type (WT) rats, homozygous  $\alpha 5$ KO rats and homozygous rats constitutively carrying the  
137 rs16969968 SNP ( $\alpha 5$ SNP rats), on Long Evans background, all males, were generated, bred and  
138 housed at the Institut Pasteur, Paris, France (Forget *et al.*, 2018). Homozygous rats were used  
139 considering the stronger phenotype on cocaine addiction in patients homozygous for the  
140 rs16969968 polymorphism (Grucza *et al.*, 2008). The rats' colonies were regularly back-crossed  
141 with WT Long-Evans rats.

142 Experimental procedures were approved by the institutional Animal Care Committee (agreement  
143 N°0355.02). All efforts were made to minimize animal suffering and reduce the number of  
144 animals.

145 **1.2.1.2 Cocaine-SA procedure**

146 Self-administration (SA) sessions were carried out in experimental chambers equipped with two  
147 retractable levers (Med Associates, St. Albans, Vt., USA), as previously described (Forget *et al.*,  
148 2009; Forget *et al.*, 2010a; Forget *et al.*, 2018; Forget *et al.*, 2010b). Cocaine hydrochloride  
149 (Sigma-Aldrich, St Louis, Mo., USA, ref: C5776) was dissolved in saline (0.9%) and  
150 administered intravenously (IV) in a volume of 100  $\mu$ l/kg/injection or subcutaneously (SC) in a  
151 volume of 1 ml/kg.

152 Rats were first trained to press an active lever (AL) under a schedule in which each AL press  
153 resulted in the delivery of a 45 mg food pellet (continuous reinforcement, no associated cues, 1h).  
154 Once trained, each animal was surgically prepared with a chronic intravenous catheter implanted  
155 in the jugular vein; the catheters exited between the scapulae. Surgery was performed under  
156 anesthesia induced by xylazine (10 mg/kg, intraperitoneal (IP)) and ketamine hydrochloride (75  
157 mg/kg, IP). Incision sites were infiltrated with the local anesthetic bupivacaine (0.125%).  
158 Buprenorphine was given for post-operative analgesia (0.01 mg/kg, SC), and a single dose of  
159 penicillin (30,000 units, SC) was administered before surgical procedures. Rats were allowed to  
160 recover for a 1-week period before drug SA sessions were begun. Every SA session lasted 1h.

#### 161 *1.2.1.2.1 Acquisition of cocaine self-administration*

162 WT (n=11),  $\alpha$ 5SNP (n=10), and  $\alpha$ 5KO rats (n=12) acquired cocaine SA under a fixed ratio (FR)  
163 schedule of reinforcement and the unit dose was 0.2 mg/kg per infusion. Each infusion was  
164 associated with the presentation of a visual cue above the AL during a time-out period of 20 sec  
165 (the levers were not retracted during the time-out period). During three consecutive sessions, each  
166 AL press during the time-in period resulted in the delivery of an infusion (FR-1). The next ten  
167 sessions, response requirements were increased to the final value of FR-5 (i.e., rats were required  
168 to make five lever presses for each drug infusion). Rats were considered to have acquired stable  
169 cocaine-SA when they pressed the AL more than twice the number of times they pressed the  
170 inactive lever (IL), and received a minimum of 5 infusions per 1-h session with less than 20%  
171 variation in the number of infusions earned per session during two consecutive sessions.

#### 172 *1.2.1.2.2 Dose-response curve*

173 The rats that had successfully acquired cocaine-SA behavior at the training dose (9 WT, 8  $\alpha$ 5SNP  
174 and 11  $\alpha$ 5KO rats) were then submitted to SA sessions with different doses of cocaine (0.1; 0.2;  
175 0.4 and 0.8 mg/kg/infusion) according to a Latin square design; with at least three sessions for

176 each dose and a stable responding required, with intermediate sessions at the 0.2 mg/kg/infusion  
177 training dose between each testing with another dose. After this testing, rats were submitted to  
178 five consecutive sessions with saline substituted to cocaine and the cocaine-associated cue still  
179 present. Cocaine-SA at the training dose was then re-established before the progressive ratio (PR)  
180 phase. Two  $\alpha$ 5KO rats were excluded from the study after this phase due to loss of catheter  
181 patency.

#### 182 *1.2.1.2.3 Progressive Ratio responding*

183 After the establishment of the dose-response curve for cocaine-SA under a FR of reinforcement,  
184 rats were switched to a PR schedule wherein the response requirement increased with each  
185 successive injection. The response requirement progression was based on the formula  
186  $5XEXP(0.2Xinfusion\ number)-5$  (Donny *et al.*, 1999). The break point (BP) was defined as the  
187 highest ratio completed prior to the first 30 min period without a response on the AL. PR sessions  
188 lasted a maximum of 3 h. Under this schedule of reinforcement, the rats were allowed to self-  
189 administer cocaine at 0.2 mg/kg/infusion until stabilization of the behavior (less than 20%  
190 variation in the number of infusions earned per session during two consecutive sessions), and  
191 then the dose was switched to 0.4 mg/kg/infusion for five consecutive sessions (9 WT, 8  $\alpha$ 5SNP  
192 and 9  $\alpha$ 5KO rats).

#### 193 *1.2.1.2.41 Reinstatement of cocaine seeking after extinction*

194 After the PR testing, the rats (9 WT, 8  $\alpha$ 5SNP and 9  $\alpha$ 5KO rats) were switched to an FR-5  
195 schedule of reinforcement (0.2 mg/kg/injection) for four consecutive sessions. Then, they were  
196 submitted to extinction sessions and reinstatement of cocaine seeking sessions induced by re-  
197 exposure to the visual cue previously associated with cocaine delivery, pharmacological stress or  
198 cocaine (10 mg/kg, SC) under a Latin square design.

199 -Extinction

200 The extinction phase was conducted by withholding cocaine and its associated cue. Responses on  
201 the AL or IL were recorded, but had no consequences. The criterion for extinction was less than  
202 20 AL presses per 1h session with less than 20 % variation over two consecutive days.

203 -Reinstatement of cocaine seeking

204 Cue-induced reinstatement test was conducted under conditions identical to that of SA, except  
205 that 1) a single presentation of the visual cue (light above the active lever for 10s) was delivered  
206 response-independently immediately at the start of the session and 2) responses on the AL  
207 resulted in contingent presentation of the cue (light above the active lever on) and house-light off  
208 for 20 s without cocaine availability (no infusions). The testing sessions lasted 1 h.

209 Pharmacological stress-induced reinstatement of cocaine seeking consisted of an IP injection of  
210 the  $\alpha$ 2-adrenergic antagonist yohimbine at 1.25 mg/kg, 30 min before the test-session. Yohimbine  
211 induces anxiety- and stress-like responses in multiple species (Bremner *et al.*, 1996a, b) and is  
212 known to reinstate cocaine seeking (Brown *et al.*, 2009; Lee *et al.*, 2004).

213 Cocaine-induced reinstatement of cocaine seeking consisted of an IP injection of cocaine at 10  
214 mg/kg, 10 min before the test-session. No cues were presented.

215 Rats were submitted again to an extinction phase between each reinstatement session.

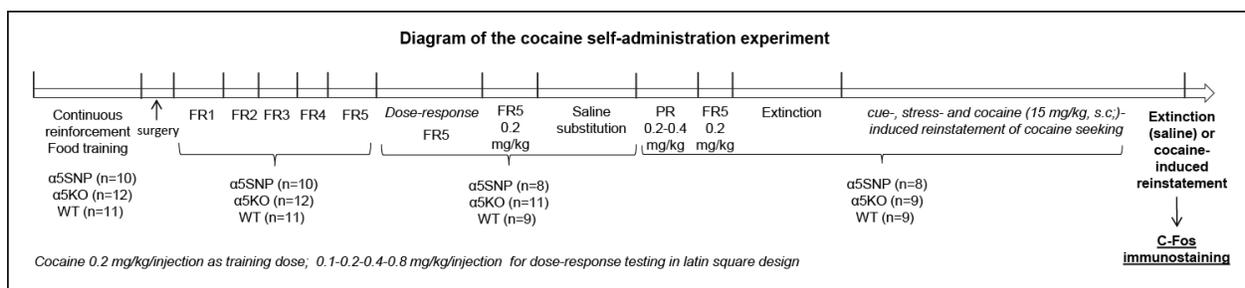
216 The extinction sessions performed before the reinstatement sessions induced by pharmacological  
217 stress or cocaine were done with saline priming injection, 30 or 10 min before the extinction  
218 session, respectively; with the same extinction criterion than during the main extinction phase.

219 The reinstatement sessions were randomized in a Latin square design.

220 *1.2.1.2.5 Statistics:*

221 Cocaine-SA data and *in vitro* and *ex-vivo* data were subjected to repeated measures ANOVAs,  
222 followed when appropriate by post hoc Bonferroni for multiple comparisons. Differences were  
223 considered significant when  $p < 0.05$ .

224 For the data on cocaine-SA under the PR schedule, the statistical analysis was made on the total  
 225 number of injections received during the session before 30 min without any AL press (BP). The  
 226 timeline of the cocaine-SA study is presented in diagram 1.



227  
 228 **Diagram 1:** Timeline of the cocaine-SA experiment. The multiples phases of cocaine-SA, doses  
 229 of cocaine and number of rats per group are presented.

230  
 231 **1.2.1.3 Immunofluorescence and c-Fos counting**

232 At the end of cocaine-induced reinstatement of cocaine seeking sessions or saline priming  
 233 extinction sessions, the rats were returned to their home cage for 60 min to allow for the synthesis  
 234 and transport of c-Fos proteins to the nuclei of activated neurons (Barros *et al.*, 2015). The rats  
 235 were then perfused transcardially with 150 mL of phosphate-buffered saline (PBS), followed by  
 236 150 mL 4% paraformaldehyde depolymerized in saline phosphate buffer. The brains were  
 237 extracted and immersed overnight in the same fixative at 4°C. Sections (50 μm) were cut  
 238 throughout the entire brain using a vibratome (Leica) and 1 slice over 3 was processed for  
 239 immunofluorescence.

240 Free-floating brain sections were incubated 1 h at room temperature in a blocking solution of  
 241 PBS containing 10% NGS and 0.2% Triton X-100 and then incubated 72h at 4°C with a rabbit  
 242 anti-c-Fos antibody (Calbiochem #PC38) at 1:15000 dilution in PBS containing 2% NGS and  
 243 0.2% Triton X-100. The sections were incubated 3 h at room temperature with Alexa fluor 594-

244 conjugated anti-rabbit secondary antibody (Jackson Immunoresearch) at 1:400 dilution in a  
245 solution of 2% NGS and 0.2% Triton in PBS and mounted using Prolong® Gold (Thermofisher  
246 scientific).

247 All the images were acquired using a nanozoomer (Hamamatsu Nanozoomer Digital Pathology,  
248 2.0 HT, its fluorescence unit option, L11600-05, and the NanoZoomer's 3-CCD TDI camera,  
249 Hamamatsu Photonics; Cellular Imaging Facility of the Institut de la Vision, Paris) and analyzed  
250 with a 20× objective.

251 The number of c-Fos positive nuclei was automatically counted with the spot detector module of  
252 ICY software (Institut Pasteur, Paris, France) as previously described (Forget *et al.*, 2018). This  
253 program makes possible to select and count cells automatically without experimenter bias (counts  
254 were conducted without knowledge of the group assignments). In each coronal section, we  
255 sampled both ipsi- and contralateral regions separately and then averaged the density of the spots  
256 for each structure per animal. The results were expressed as number of c-Fos positive cells per  
257 square millimeter of cerebral tissue.

258 Data were analyzed using Prism and statview softwares. Data from each brain area were  
259 subjected to two-way repeated measures ANOVAs, followed when appropriate by post hoc  
260 Bonferroni for multiple comparisons. Changes were considered significant when  $p < 0.05$ .

261

#### 262 **1.2.1.4 Two-electrodes voltage-clamp electrophysiology**

263 *Xenopus laevis* oocytes were obtained from Centre de Ressources Biologiques-Rennes, France  
264 and maintained in modified Barth's medium (88mM NaCl, 1mM KCl, 1mM MgSO<sub>4</sub>, 2.5 mM  
265 NaHCO<sub>3</sub>, 0.7mM CaCl<sub>2</sub>, 5mM HEPES pH 7.3). Defolliculated oocytes were submitted to

266 intranucleus injection of a 1:1:0 or 1:1:5 mixture of  $\alpha 3:\beta 4:\alpha 5$  or  $\alpha 4:\beta 2:\alpha 5$  rat cDNAs and kept at  
267 18°C for 1-2 days before recording.

268 Recordings were performed with a Digidata 1550A (Molecular Devices) digitizer, an Oocyte  
269 Clamp OC-725C (Warner Instruments) amplifier and using the pClamp 10.5 software. Oocytes  
270 were perfused with recording buffer (100mM NaCl, 2.5mM KCl, 2mM CaCl<sub>2</sub>, 1mM MgCl<sub>2</sub>,  
271 10mM HEPES pH 7.3) using a gravity-driven system. Cocaine and acetylcholine were applied  
272 after dilution in recording buffer immediately before recording. All currents were measured at -  
273 60 mV.

274 The protocol consisted of a co-application of the given dose of cocaine and 30 $\mu$ M of  
275 acetylcholine in between two applications of 30 $\mu$ M acetylcholine. Data are from  $n \geq 3$  oocytes  
276 from at least two injections. Normalized peak current are presented  
277 ( $I_{\text{peak}}(\text{ACh+cocaine})/I_{\text{peak}}(\text{ACh})$ ). Recordings were analyzed using ClampFit, AxoGraph X  
278 and GraphPad Prism.

279

#### 280 **1.2.1.5 Clinical genetic association study**

281 This was both a within-cases and case/control, candidate SNP, preclinically-driven genetic  
282 association study.

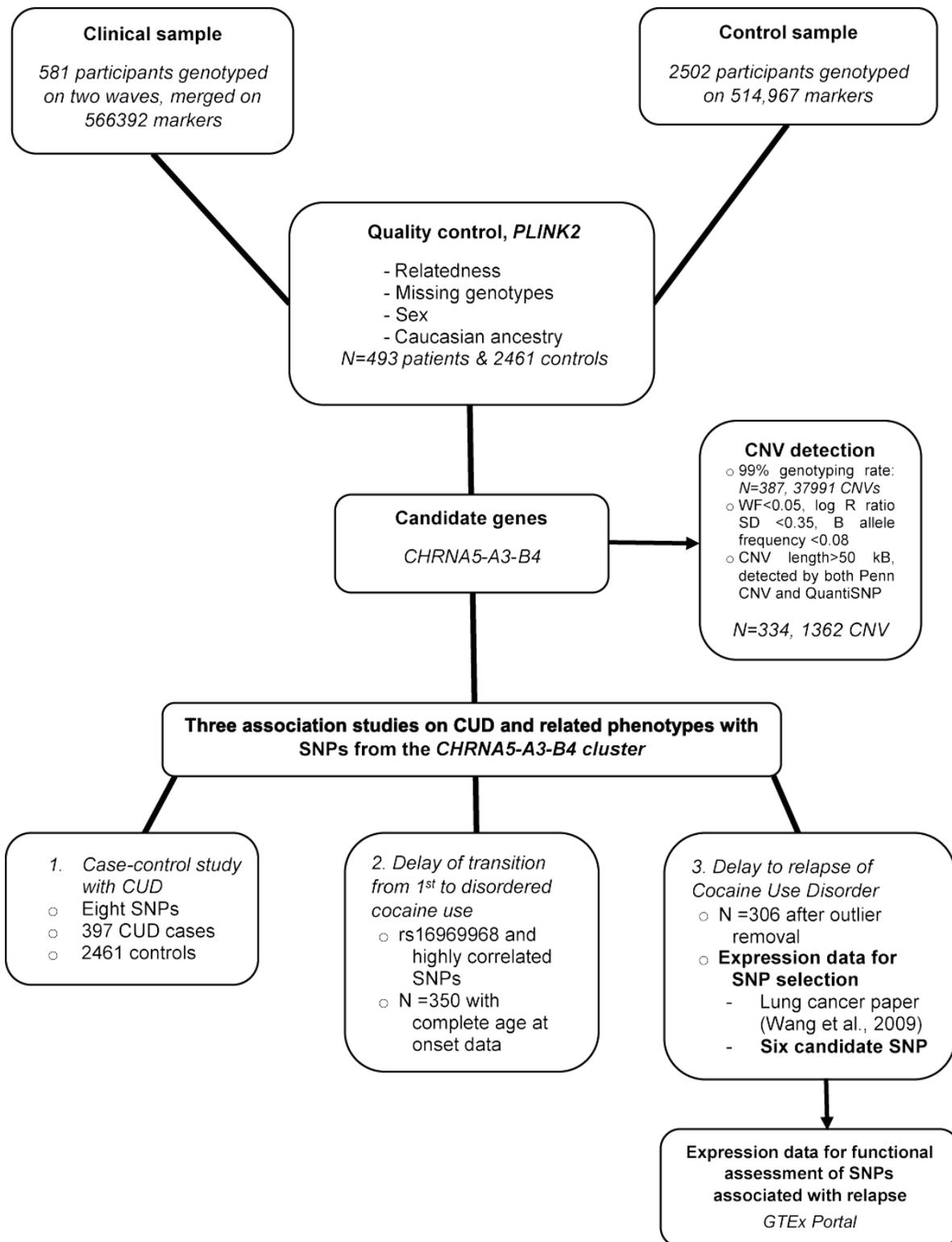
283 Study protocol was approved by *INSERM* institutional review board (IRB00003888) in July  
284 2015.

285 Participants were genotyped using the Infinium PsychChip array (Illumina, San Diego, CA,  
286 USA). Samples were processed by Integragen SA® (Evry, France).

287 *PLINK* software (Chang *et al.*, 2015) was used for quality control, based on a consensus  
288 procedure (Marees *et al.*, 2018) (**Diagram 2, Fig. S2**), which left 21 SNPs from the chromosome

289 15 *CHRNA5-A3-B4* cluster for analysis (**Fig. S2**). The candidate SNPs for each study were  
290 selected based on previous literature (case/control analysis) and the preclinical results ( $\alpha$ 5SNP for  
291 CUD development, modifiers of *CHRNA5* expression for relapse). We evaluated:

- 292 - The association of eight SNPs from the *CHRNA5-A3-B4* cluster, that could be analyzed  
293 with high confidence (quality >90%) after imputation of chromosome 15 SNPs using the  
294 best practice pipeline (pre-phasing with SHAPEIT v.2 then IMPUTE2), with the presence  
295 of lifetime CUD (*N=397 patients with CUD compared to N=2461 controls genotyped at  
296 the Wellcome Trust Institute, case-control study*).
- 297 - The association between *CHRNA5* rs16969968 and SNPs from *CHRNA5-A3-B4* cluster in  
298 high LD, with the time to transition from 1<sup>st</sup> to disordered cocaine use (*N=350 with CUD,  
299 survival model*).
- 300 - The association of *CHRNA5* expression-modifying SNPs from *CHRNA5-A3-B4* cluster  
301 associated with the delay to relapse to cocaine use after cessation (*N =306 after outlier  
302 removal, survival model*).



303

304 **Diagram 2:** Flowchart of patients and single nucleotide polymorphism (SNP) selection according  
 305 to ancestry, quality control, and expression data for *CHRNA5*. SNP, single nucleotide  
 306 polymorphism; CUD, cocaine use disorder.

307 *1.2.1.5.1 Sample selection and clinical assessment*

308 Patients > 18 years seeking treatment for any substance use disorder (SUD) in any of the  
309 participating Centre, and fulfilling DSM IV-TR criteria for either lifetime cocaine or opiate use  
310 disorder, were consecutively recruited through two multicentric protocols (Mouly *et al.*, 2015;  
311 Vorspan *et al.*, 2015) aimed at deciphering the phenotypic and genotypic architecture of severe  
312 SUDs (Icick *et al.*, 2017), between April 2008 and July 2016. They provided written informed  
313 consent for both the clinical and genetic assessments. Study protocol was approved by *INSERM*  
314 institutional review board (IRB00003888) in July 2015. Eligible participants were excluded if  
315 they had severe cognitive impairment or insufficient mastery of the French language preventing  
316 their proper understanding of the study purposes and assessments, had no social insurance, or  
317 were under compulsory treatment. A unique standardized interview conducted by trained  
318 investigators (psychologists or M.D) allowed for collecting extensive phenotypic data regarding  
319 participants' history of SUD with the *Structured Clinical Interview for DSM-IV* (SCID-IV)  
320 (First, 1997) to ascertain DSM-IV lifetime diagnoses of substance abuse and dependence, along  
321 with enhanced information regarding the participants' modalities and trajectory of drug use and  
322 sociodemographic data. Investigators used standard survey tips to determine these events with the  
323 best possible accuracy (Kendig *et al.*, 2014).

324 The main phenotype of interest was the delay to relapse to cocaine use after cessation. It was  
325 obtained by 1/ asking cocaine use disorder (CUD) participants whether they had ever stopped  
326 using cocaine for more than one day, 2/ by checking whether these 'ever-stoppers' were still not  
327 using cocaine in the past 30 days and 3/ among those who ever relapsed, estimating the delay – in  
328 months – between cocaine cessation and relapse. The measure was right-censored by the  
329 interview (*i.e.* non-relapsers at that time could still relapse after the study ended) and was thus  
330 analyzed using survival models (see *Statistical Analyses* subsection). We also considered the

331 presence of CUD and the time to transition from 1<sup>st</sup> to disordered cocaine use as our secondary  
332 phenotypes of interest (Fig.3).

#### 333 *1.2.1.5.2 Biological sampling*

334 Participants' DNA was extracted from whole blood using a Maxwell 16 PROMEGA® extractor  
335 (Promega France, Charbonnières-les-Bains, France). Purity assessment followed the procedures  
336 described by the Centre National de Génomique, estimated on a NanoDrop® spectrophotometer  
337 by using Picogreen® assay, confirmed by Polymerase Chain Reaction before application on gel.  
338 Participants were genotyped using the Infinium PsychChip array (Illumina, San Diego, CA,  
339 USA). Samples were processed in two stages (2014 and 2017) by Integragen SA® (Evry, France)  
340 and genotype files were merged for the present study, keeping only bi-allelic variants common to  
341 both extractions.

#### 342 *1.2.1.5.3 Bioinformatic quality control for genetic analyses*

343 *PLINK* software (Chang *et al.*, 2015) was used for quality control, based on a consensus  
344 procedure (Marees *et al.*, 2018) (see flowchart, **Diagram 2**). At the whole-sample (N=581) and  
345 genome-wide (566,932 single nucleotide variants (SNVs)) levels, SNVs and individuals with >  
346 2% genotype missingness were excluded. Sex discrepancy was also verified, and only SNVs with  
347 minor allele frequency (MAF)  $\geq 5\%$  (thus targeting single nucleotide polymorphisms, hereafter  
348 referred to as SNPs), and without deviation from Hardy-Weinberg Equilibrium at  $p < 10^{-6}$  were  
349 analyzed, leaving a total of 579 individuals and 260,853 markers. Then, cryptic relatedness by  
350 Identity-by-Descent was searched for, using a pi-hat threshold of 0.2 (2<sup>nd</sup> degree relatedness), and  
351 Caucasian ancestry was assessed according to their genetic proximity with known Caucasian  
352 populations described in the 1000 genomes project (1000G) panel, based on visual inspection of  
353 principal component analysis (N=2502 from five superpopulations). As a result, 31 individuals  
354 with the lowest genotyping rates from 62 pairs (58 pairs were duplicate inclusions across original

355 protocols, two participants were known brothers and two were unknown cousins according to  
356 piHat scores) and another 75 individuals due to non-Caucasian ancestry were excluded. After  
357 these procedures, 21 SNPs from the *CHRNA5-A3-B4* cluster on chromosome 15 were identified  
358 on the DNA array for further analysis (**Fig. S2**).

#### 359 *1.2.1.5.4 Control sample*

360 The dataset *EGA 000100000290* was downloaded from the Wellcome Trust initiative through a  
361 registered procedure from a dedicated website. It consists of 2502 UK blood donors genotyped on  
362 the Affymetrix-500k® DNA array. These subjects underwent the same stringent quality control  
363 as clinical cases. At the whole-sample (N=2502) and genome-wide (514,967 single nucleotide  
364 variants (SNVs)) levels, 1546 SNVs and 0 individual with > 2% genotype missingness were  
365 excluded; one due to sex discrepancy. One SNVs with deviation from Hardy-Weinberg  
366 Equilibrium at  $p < 10^{-6}$  was removed. Thirty individuals were excluded for having a  
367 heterozygosity rate deviating more than 3 standard deviations from the mean. Then, cryptic  
368 relatedness by Identity-by-Descent was searched for, using a pi-hat threshold of 0.2 (2nd degree  
369 relatedness), and Caucasian ancestry was assessed according to their genetic proximity with  
370 known Caucasian populations described in the 1000 genomes project (1000G) panel, based on  
371 visual inspection of principal component analysis (N=2504 from five superpopulations). Of note,  
372 the first two components of this “MDS” analysis were kept as covariates for the case/control  
373 analysis, as recommended. These procedures led to exclude four individuals with non-Caucasian  
374 ancestry and six due to relatedness. After these procedures, 2461 people and 488479 variants  
375 remained.

#### 376 *1.2.1.5.5 Imputation*

377 We followed the procedure described on the Oxford University dedicated website  
378 ([https://mathgen.stats.ox.ac.uk/impute/impute\\_v2.html](https://mathgen.stats.ox.ac.uk/impute/impute_v2.html)). The clinical cases with CUD and the

379 control samples were merged on common, bi-allelic variants with a minor allele frequency >5%  
380 using PLINK *ad hoc* function, leaving 239,578 shared SNPs. After resolving strand issues using  
381 the 1000 genomes, phase 3 reference panel for chromosome 15 (and the corresponding genetic  
382 map, version b37) (Sudmant *et al.*, 2015), pre-phasing was performed using SHAPEIT (v2)  
383 (Delaneau *et al.*, 2014) using default parameters. Imputation for the 21 SNPs identified in the  
384 clinical samples was performed using IMPUTE2 (Howie *et al.*, 2009) on chromosome 15 interval  
385 between 78.5 and 79.1 M base pairs, using default parameters except for a buffer of 500 K base  
386 pairs, setting the number of template haplotypes to 100 and the addition of the same reference  
387 panel (1000 genomes phase 3) to the pre-phased genotypes. Minimal concordance was 88%, and  
388 certainty was above 0.995 regarding the SNPs, which were further analyzed.

#### 389 *1.2.1.5.6 Case-control analysis*

390 Analysis was performed with PLINK on 397 cases and 2461 controls using sex and the first two  
391 components of the MDS (ancestry) analysis as covariates. Bonferroni correction was applied.  
392 Considering 3% lifetime prevalence of CUD in the general population and the odds ratio of  
393 rs16969968 risk allele for this disorder, power calculation (Sham and Purcell, 2014) considering  
394 unselected controls yielded  $\beta = 80\%$  for this analysis ( $p$ -value after Bonferroni correction  
395  $= 0.05/16 = 0.003125$ ).

#### 396 *1.2.1.5.7 Impact of CHRNA5 rs16969968 on the transition from 1<sup>st</sup> to disordered cocaine use*

397 In the preclinical experiment, rats carrying the  $\alpha 5$ SNP showed altered acquisition of cocaine SA.  
398 To investigate this phenomenon in the clinical sample, we tested the association between  
399 *CHRNA5* rs16969968 and SNPs from *CHRNA5-A3-B4* cluster in high linkage disequilibrium  
400 (LD) ( $r^2 \geq 0.8$ , *CHRNA3* rs12914385 and *CHRNA4* rs950776) with the time to transition from 1<sup>st</sup>  
401 to disordered cocaine use, using a survival model among CUD individuals only (since rats had all

402 acquired the self-administration behavior in the preclinical study). Tests were performed under  
403 both the genotypic (log-rank test for trend) and the recessive models (binary log-rank test). Five  
404 patients were excluded from analysis due to extreme values of the transition duration ( $\geq 300$   
405 months, three of whom were major allele homozygotes), leaving 351 CUD individuals with valid  
406 data regarding their age at onset of cocaine experimentation and of CUD. *P*-values were  
407 Bonferroni-corrected for three tests (cut-off  $=0.05/3 = 0.016$ ). Significant associations were  
408 further confirmed after multiple adjustment on clinical and sociodemographic variables also  
409 associated with the time to transition from 1<sup>st</sup> to disordered cocaine use under the recessive model  
410 using cox model, after verification of the proportionality of hazard ratios.

#### 411 1.2.1.5.8 Genetic study of relapse to CUD

412 SNPs from *CHRNA5-A3-B4* cluster were further filtered and selected according to their effect on  
413 *CHRNA5* expression, to perform a candidate association study with relapse to cocaine use in  
414 treatment-seeking outpatients. This selection was based on data from an extensive *post-mortem*  
415 study (Wang *et al.*, 2009) reporting *CHRNA5* mRNA levels according to 44 SNPs from  
416 *CHRNA5-A3-B4* cluster in their Table 1. We applied Bonferroni correction to these 44 SNPs, of  
417 which 28 remained significantly associated with *CHRNA5* mRNA levels. Eventually, six of these  
418 SNPs were available on our DNA array and could be tested for associations with the delay to  
419 relapse to cocaine use in our sample (**Diagram 2**).

#### 420 1.2.1.5.9 Copy Number Variants (CNVs)

421 We followed the pipeline used in a recent study (Huguet *et al.*, 2018), starting from Illumina®  
422 final report file generated by *GenomeStudio* v.2, that involves two detection programs, *PennCNV*  
423 (Wang *et al.*, 2007) and *QuantiSNP* (Colella *et al.*, 2007) optimized in a combined algorithm  
424 allowing for thorough quality check. Both are based on hidden Markov chains model, but show  
425 different sensitivity and specificity. The aim of such analysis was to identify additional variants

426 that would be relevant to fuel further *in vitro* and *in vivo* functional analyses. It was thus  
427 performed at the genome-wide and sample-wide levels. We only considered individuals and CNV  
428 meeting stringent control criteria (**Diagram 2**).

#### 429 1.2.1.5.10 Statistical analyses

430 Data is described by means (standard deviation, SD) or median (interquartile range, IQR) when  
431 continuous and counts (frequencies) when categorical.

432 In the clinical sample, non-parametric  $\chi^2$ , Spearman's *rho* and Mann-Whitney *U* were used to  
433 identify the sociodemographic and clinical variables associated with the three phenotypes of  
434 interests. Of note, both the delay to transition and the delay to relapse had a highly skewed  
435 distribution with Shapiro-Wilk test indicating lack of normality at  $p < 2.2 \times 10^{-16}$ . The variables  
436 were thus log-transformed for bivariate analyses. Before fitting survival models, multivariate  
437 identification of outliers was also conducted. It was deemed relevant because 1/ the distribution  
438 was highly skewed (**Fig. S1**) and 2/ some respondents reported delays to relapse as low as a few  
439 days, which – although it was not corrected by the interviewer in order to maintain a fully  
440 naturalistic and observational study design – should be considered as continuous use or  
441 withdrawal relief rather than relapse. To do so, the *cook.distance* function was applied to the  
442 linear fit of the log-transformed delay variables and the most influential values were removed  
443 (Cook and Weisberg, 1982).

444 Across all human genetic studies, ancestry was thoroughly controlled for, since the minor allele  
445 frequency of the  $\alpha 5$ SNP (rs16969968) ranges from in 2.3% among African-Americans to 36.6%  
446 in Europeans. Thus, mixing these populations would deeply bias the interpretation of group  
447 comparisons.

448 Cox proportional models and curves were built separately for each SNP associated with the  
449 delays of transition and relapse. After verification of the proportional hazard assumption, hazard  
450 ratios (HR) were computed to detect significant differences in those delays according to binary  
451 genotypes under the recessive model (major allele homozygotes vs. carriers of any number of  
452 minor allele copies), considered significant at Bonferroni-corrected  $p=0.05$ . In case HR were  
453 significantly associated with genotypes, clinical and sociodemographic correlates of the given  
454 delay (transition or relapse) identified in bivariate analyses were incorporated in final cox models  
455 to test the independence of genetic associations. Analyses were performed by R 3.5.3 (through R  
456 studio 1.185) on Mac OS X.12.6.

457 *1.2.1.5.11 “Weighted” NES calculation:* in order to test if the genotyped SNPs possibly  
458 associated with CUD relapse yielded a reduction of *CHRNA5* expression, i.e. if they would  
459 mimic the results from the preclinical study, we investigated the effects of both genotyped and  
460 non-genotyped SNPs known to be coinherited SNPs with our lead SNP(s). To do so, we searched  
461 gene expression data from the *GTE*x portal (available at <https://gtexportal.org>), which is based on  
462 actual gene expression measurements in multiple tissue samples (Baran *et al.*, 2015), using a LD  
463 threshold of  $r^2 > 0.6$ , considering the signed normalized effect size (NES) of each SNP  
464 significantly associated with *CHRNA5* expression in eight brain regions. There were a total of  
465 311 variants in high LD in the *CHRNA5-CHRNA4-CHRNA3* cluster. LD scores of all SNPs with  
466 available and significant association with *CHRNA5* expression were used as coefficients to  
467 weight each NES, so that  $final\ NES = LD_1 * NES_1 + LD_2 * NES_2 + \dots + LD_i * NES_i$ . We also searched for  
468 associations between our lead SNP(s) and DNA methylation of *CHRNA5* in the mQTLdb  
469 database (available at <http://www.mqtlldb.org>), which is also based on actual cellular  
470 experimentation (Gaunt *et al.*, 2016).

471 *Limitations:* Our Human genetic study shows some limitations. In particular, they have been  
472 performed on a limited set of SNPs. However, selection was driven by results from our  
473 preclinical study and from reliable expression data in the literature. The model used to estimate  
474 the effects of rs950776 on *CHRNA5* expression was purely empirical and intended for discussion  
475 purposes only. Binary genotypes under the recessive model were mostly considered, as  
476 previously published (Grucza *et al.*, 2008). Accordingly, direct causality cannot be drawn from  
477 the present human association studies results.

478

## 479 **1.3 Results**

### 480 **1.3.1 Cocaine-SA and relapse in transgenic rats**

481

#### 482 *1.3.1.1 Acquisition of cocaine-SA, dose-response curve, saline substitution and motivation for* 483 *cocaine-SA in $\alpha$ 5SNP, $\alpha$ 5KO and WT rats*

484  $\alpha$ 5SNP,  $\alpha$ 5KO and WT rats acquired the food training similarly (continuous reinforcement, no  
485 associated cues; **Fig. 1A**), suggesting an absence of learning or motor deficit in  $\alpha$ 5SNP and  $\alpha$ 5KO  
486 rats.

487 Rats were then submitted to chronic IV cocaine-SA. The three groups increased similarly their  
488 lever pressing behavior when the ratio progressively increased (FR-1 then FR-2 then FR-3 then  
489 FR-4 and finally FR-5; **Fig. 1B**). However, there was a significant interaction between sessions  
490 and genotypes on the number of cocaine infusions received during cocaine-SA acquisition (**Fig.**  
491 **1C**), indicating that the evolution of SA acquisition was different between groups. Indeed, there  
492 was a session effect only in  $\alpha$ 5SNP rats, showing that they were unable to maintain their level of  
493 cocaine intake when the ratio increased, contrary to WT and  $\alpha$ 5KOs. In addition, the mean

494 number of cocaine infusions received during the fixed-ratio 5 (FR-5) sessions was significantly  
495 reduced compared to FR-1 sessions in  $\alpha$ 5SNP rats but not in WT or  $\alpha$ 5KOs (**Fig. 1D**),  
496 confirming the deficit in cocaine-SA acquisition of  $\alpha$ 5SNP rats. There were no significant  
497 differences between groups on inactive lever (IL) presses or on perseverative responding during  
498 the time-out period (wasted presses) (**Table S1**) or on the number of sessions necessary to reach  
499 the criterion (**Table S2**).

500  
501  $\alpha$ 5SNP,  $\alpha$ 5KO and WT rats were then allowed to self-administer multiple doses of cocaine  
502 according to a Latin-square design (0.2-0.8 mg/kg/injection) under FR-5, to establish a dose-  
503 response curve. We did not observe significant differences on the number of cocaine infusions  
504 received during these sessions between groups (**Fig. 1E**). There were also no differences between  
505 groups on IL or wasted presses (**Table S1**). The fact that the deficit in cocaine-SA acquisition of  
506  $\alpha$ 5SNP rats was no longer present at the same dose (0.2 mg/kg) throughout the dose effect  
507 investigation suggest that the protective effect of the  $\alpha$ 5SNP is temporary and restricted to an  
508 early phase of cocaine-SA.

509 When saline was substituted for cocaine during five consecutive sessions,  $\alpha$ 5SNP,  $\alpha$ 5KO and WT  
510 rats displayed similar AL pressing extinction (**Fig. 1F**), indicating that cocaine expectation drives  
511 the lever pressing behavior.

512 We then shifted the schedule of reinforcement to a progressive ratio (PR) (Donny *et al.*, 1999),  
513 allowing the measurement of the incentive motivational and rewarding properties of cocaine  
514 (Paterson and Markou, 2005). We found no significant differences between genotypes for 0.2 and  
515 0.4 mg/kg of cocaine, i.e. on the number of reinforcements received during the test (**Fig. 1G**), nor  
516 on IL or wasted presses (**Table S1**).

517 An extinction phase was then conducted by withholding cocaine and its associated visual cue,  
518 inducing a progressive and similar decrease of AL responding in the three groups (**Fig. 1H**),  
519 without a difference in the number of sessions necessary to reach the extinction criterion (**Table**  
520 **S2**).

521

### 522 *1.3.1.2 Reinstatement of cocaine seeking after extinction in $\alpha 5$ SNP, $\alpha 5$ KO and WT rats*

523 Rats were then tested for reinstatement of cocaine seeking induced by cue, pharmacological  
524 stress or cocaine in a Latin-square design. WT,  $\alpha 5$ SNP and  $\alpha 5$ KO rats exhibited similar cue- and  
525 stress-induced reinstatement of cocaine seeking (**Figs. 2A and B**, respectively). However, we  
526 observed a strong increase in cocaine-induced reinstatement of cocaine seeking in  $\alpha 5$ KO rats  
527 compared to WTs and  $\alpha 5$ SNPs (**Fig. 2C**), without modifying IL pressing behavior (**Table S1**).  
528 This indicates that the absence of nAChR  $\alpha 5$  subunit, and not  $\alpha 5$ SNP presence, leads to higher  
529 level of relapse after re-exposure to cocaine, but not to cocaine-associated cue nor after exposure  
530 to stress.

531

### 532 *1.3.1.3 Neuronal activation during cocaine-induced reinstatement of cocaine seeking*

533 To dissect the effect of the absence of the  $\alpha 5$  subunit on relapse, we investigated the expression  
534 of the c-Fos immediate early gene product, considered as a marker of neuronal activation (Forget  
535 *et al.*, 2010a), during cocaine-induced reinstatement of cocaine seeking or extinction session with  
536 saline injection, in multiple brain areas of WT and  $\alpha 5$ KO rats. Cocaine-induced reinstatement of  
537 cocaine seeking was associated with an increase of c-Fos expression in WT and  $\alpha 5$ KO rats  
538 compared to saline condition in prelimbic, infralimbic and cingulate cortices, in the shell and core  
539 of the NAcc, the ventral tegmental area (VTA), the dorsal striatum and the primary motor cortex  
540 (M1) (**Fig. 2D**). Interestingly, a significantly higher expression of c-Fos was observed in the shell

541 of the NAcc of  $\alpha 5$ KO rats, which relapsed more intensely compared to WT, during cocaine-  
542 induced relapse but not extinction.

543  
544 **1.3.2 Impact of the  $\alpha 5$  subunit on cocaine-induced inhibition of cholinergic currents on**  
545  **$\alpha 3\beta 4$ - or  $\alpha 4\beta 2$ -containing nicotinic receptors in oocytes**

546 Since biologically relevant concentrations of cocaine can inhibit nAChR-mediated currents in  
547 midbrain dopaminergic neurons, leading to an alteration of dopamine release in the dorsal and  
548 ventral striatum (Acevedo-Rodriguez *et al.*, 2014); we next investigated whether the increased  
549 cocaine-induced relapse observed in  $\alpha 5$ KO rats may be due to an alteration of cocaine-induced  
550 inhibition of nAChRs in the absence of  $\alpha 5$ . To do so, we measured cholinergic currents in  
551 *Xenopus* oocytes expressing  $\alpha 3\beta 4$ - or  $\alpha 4\beta 2$ -nAChRs in the presence or absence of  $\alpha 5$ . As  
552 expected, we observed a dose-dependent inhibition of cholinergic currents in oocytes expressing  
553  $\alpha 3\beta 4$ - or  $\alpha 4\beta 2$ -nAChRs by cocaine (0-100  $\mu$ M) (**Fig. S3A and B, left panels**). However, the  
554 addition of the  $\alpha 5$  subunit did not modify the effect of cocaine on either nAChR subtypes (**Fig.**  
555 **S3A and B, right panels**), suggesting that  $\alpha 5$  does not contribute directly to cocaine-induced  
556 inhibition of nAChR currents, at least in this heterologous expression system.

557  
558 **1.3.3 Genetic association study in patients**

559 We then sought to establish the role of  $\alpha 5$ SNP and *CHRNA5* expression levels in CUD patients.  
560 The flowchart of the study is described in **Diagram 2** and the sample is described in **Table S3**.  
561 Patients were 39 +/- 9 years old, 76% men, with a median delay to CUD relapse of 13 months,  
562 the distribution of which was highly skewed (**Fig. S1**). There were no significant associations  
563 among the eight SNPs from the *CHRNA5-A3-B4* cluster and the presence of lifetime CUD (**Table**  
564 **1**). In particular, we did not replicate the association between *CHRNA5* rs16996698 SNP and

565 CUD (Grucza *et al.*, 2008; Sherva *et al.*, 2010). However, *CHRNA5* rs16969968 was significantly  
566 associated with a slower transition from 1<sup>st</sup> to disordered cocaine use. Under the recessive model,  
567 hazard ratio (HR) was 0.73 (95% CI =0.58-0.91, corrected  $p =0.014$ , **Fig. 3A**) for carriers of at  
568 least one copy of the minor allele. *CHRNA3* rs12914385 and *CHRNA4* rs950776, which are in  
569 high LD with rs16969968, were not significantly associated with transition from 1<sup>st</sup> to disordered  
570 cocaine use [corrected  $p$ -values of 0.2 for both SNPs under the genotypic model; and 0.063  
571 (rs12914385) and 0.26 (rs950776) under the recessive model]. The association withheld  
572 adjustment on gender, protocol of origin, preferred route of cocaine administration during the  
573 self-reported worst period, the number of DSM-IV criteria for CUD, and the presence of opiate  
574 use disorder (**Fig. 3B**; only significant associations are shown). The adjusted HR was 0.73 (95%  
575 CI =0.59-0.92,  $p =0.007$ ) for carriers of at least one copy of the *CHRNA5* rs16969968 as  
576 compared to major allele homozygotes.

577 Considering the increased cocaine-induced relapse intensity observed in  $\alpha 5$ KO rats during the SA  
578 experiment, genotyped SNPs from the *CHRNA5-A3-B4* cluster were further filtered by their  
579 effect on *CHRNA5* expression in human brain (Wang *et al.*, 2009) and selected to perform a  
580 candidate association study with relapse to cocaine use in the CUD patients. Among the six final  
581 SNPs that were selected based on their previously reported effect on *CHRNA5* mRNA  
582 expression, five were intron variants and one (rs3743074) was a splice region variant.

583 One SNP, rs950776 in *CHRNA4*, was significantly associated with a shorter delay to relapse to  
584 cocaine use (HR = 1.58, 95% CI=1.41-1.75, Bonferroni-corrected  $p =0.04$ ) (**Fig. 3C**). Shorter  
585 delay to relapse was also associated with a higher number of DSM-IV criteria for cocaine  
586 abuse/dependence (HR=1.41, 95% CI =1.27-1.58,  $p <0.001$ ) while both alcohol (HR=0.58, 95%  
587 CI=0.4-0.86,  $p=0.006$ ) and sedative medication (HR =0.69, 95% CI=0.48-0.98,  $p=0.037$ ) use

588 disorders were independently associated with a longer delay to relapse to cocaine (**Fig. 3D**). No  
589 CNVs were identified in the *CHRNA5-A3-B4* cluster.

590

## 591 **1.4 Discussion**

### 592 *1.4.1 Phenotypes of $\alpha 5$ SNP and $\alpha 5$ KO rats in cocaine-SA and relapse*

593 We first observed that the presence the  $\alpha 5$ SNP did not prevent rats from acquiring cocaine-SA,  
594 and did not alter the dose-response curve for cocaine-SA. Yet, contrary to WTs,  $\alpha 5$ SNP rats did  
595 not adapt their AL pressing to maintain constant amounts of cocaine intake when the ratio  
596 schedule of reinforcement was increased (**Fig. 1C and D**). This seems in line with previous  
597 indications of a protective effect of this polymorphism on cocaine dependence in humans (Grucza  
598 *et al.*, 2008; Sherva *et al.*, 2010). However, the deficit in cocaine-SA acquisition of  $\alpha 5$ SNP rats  
599 was no longer present at the same dose after extensive SA sessions, and we further found that the  
600  $\alpha 5$ SNP did not alter cocaine-SA extinction and relapse to cocaine seeking induced by cocaine-  
601 cue, pharmacological stress or cocaine priming. These results suggest that this polymorphism  
602 protects against the risk to develop cocaine abuse through altered cocaine effects during early  
603 stages of drug experimentation, but has no longer an impact on later stages of the disease when  
604 CUD is already installed.

605 The absence of the  $\alpha 5$  nAChR subunit had no effect on cocaine-SA acquisition and dose-response  
606 curve, nor on the extinction or reinstatement of cocaine seeking induced by cocaine-cue or  
607 pharmacological stress. However, KO rats showed a strong increase in relapse intensity induced  
608 by cocaine priming. This constitutes an important finding considering the high frequency of  
609 relapse in cocaine addiction (Sinha, 2011) and is in line with the fact that three SNPs in the  
610 *CHRNA5* gene previously associated with cocaine dependence (rs684513, rs621849 and  
611 rs615470 (Sherva *et al.*, 2010)) are also associated with modifications in *CHRNA5* mRNA

612 expression in the human brain (Wang *et al.*, 2009). It would now be interesting to evaluate  
613 whether these SNPs specifically affect the risk to relapse to cocaine seeking in humans.

614 Additional work will also now be needed to further understand how the  $\alpha 5$ SNP, which has been  
615 proposed to confer a partial loss of function of the receptor although the underlying mechanisms  
616 are still unclear (Maskos, 2020; Prevost *et al.*, 2020), may confer protection against CUD  
617 development by modifying the effects of cocaine at early stages of exposure, while a full absence  
618 of  $\alpha 5$ nAChRs rather seems to increase the risk to relapse after prior long-term exposure.

#### 619 ***1.4.2 Neuronal activation during cocaine-induced relapse***

620 We also found that cocaine-induced reinstatement of cocaine seeking was associated with  
621 increased neuronal activation in WT and  $\alpha 5$ KO rats, in most of the brain areas investigated: the  
622 prelimbic, infralimbic and cingulate cortices, the shell and core of the NAcc, the posterior VTA,  
623 the dorsal striatum and the primary motor cortex (M1). This is in line with a previous study  
624 showing an increase in c-Fos expression in the NAcc and prefrontal cortex during cocaine-  
625 induced reinstatement of cocaine seeking in the SA paradigm (Wright *et al.*, 2015), and adds the  
626 cingulate cortex, the posterior VTA, the dorsal striatum and M1 to the list of brain areas activated  
627 during this behavior.

628 Interestingly, only the shell of the NAcc was associated with a significant increase in neuronal  
629 activation in  $\alpha 5$ KO rats compared to WT. However, we observed similar tendencies in the  
630 prelimbic, infralimbic and cingulate cortices and in the NAcc core, all part of the neuronal  
631 ensemble involved in relapse to drug seeking (Cruz *et al.*, 2015). It is thus possible that the  
632 activation of these brain areas is also affected by the absence of the  $\alpha 5$  subunit but that the  
633 present analysis is not powerful enough to detect it. Interestingly, we did not observe any  
634 difference or tendency between  $\alpha 5$ KO and WT rats on c-Fos expression in M1, a structure mainly

635 involved in motor control and procedural skill learning (Papale and Hooks, 2018); suggesting that  
636 the increase in neuronal activation in  $\alpha 5$ KO rats is not due to a non-specific motor effect. The  
637 NAcc shell is crucial for cocaine-induced relapse since infusion of cocaine into this structure is  
638 sufficient to reinstate cocaine seeking after extinction in rats (Reed *et al.*, 2015), while inhibition  
639 of D1-like dopamine receptor in the NAcc shell attenuates cocaine priming-induced reinstatement  
640 of cocaine seeking (Anderson *et al.*, 2003). In addition, stimulation of dopamine receptors in the  
641 shell, but not the core, of the NAcc reinstates cocaine seeking (Schmidt *et al.*, 2006). Finally,  
642 MRI studies found that NAcc activity was positively correlated with craving in subjects with  
643 CUD exposed to cocaine (Breiter *et al.*, 1997; Risinger *et al.*, 2005).

644  $\alpha 5$ nAChRs are present in dopaminergic cell bodies in the VTA (Azam *et al.*, 2002) and in  
645 dopaminergic terminals in the striatum (Zoli *et al.*, 2002). Since the absence of  $\alpha 5$  can modify  
646 the pharmacological properties, calcium permeability and acetylcholine sensitivity of nAChRs  
647 (Kuryatov *et al.*, 2008; Ramirez-Latorre *et al.*, 1996; Sciacaluga *et al.*, 2015), it may also alter  
648 the inhibition of nAChRs caused by cocaine, thereby contributing to enhanced relapse to cocaine  
649 seeking in  $\alpha 5$ KO rats. However, we did not observe any difference in the presence or absence of  
650  $\alpha 5$  on cocaine-induced inhibition of cholinergic currents in oocytes expressing either  $\alpha 3\beta 4$ - or  
651  $\alpha 4\beta 2$ -containing nAChRs, at cocaine concentrations comparable to plasma level in rats after IV  
652 SA (Booze *et al.*, 1997). This suggests that the increased cocaine-induced relapse in  $\alpha 5$ KO rats is  
653 not mediated by a direct effect on cocaine-induced nAChR inhibition. Nevertheless, this may  
654 differ *in vivo* since the absence of  $\alpha 5$  may modify the interaction of nAChRs with intracellular  
655 partners (Kabbani *et al.*, 2007), which are not present in our heterologous expression system.

656 More work will now be needed to support this hypothesis. Importantly, the  $\alpha 5$ SNP was  
657 previously identified as predisposing to increased addiction-like behaviors for both nicotine  
658 (Forget *et al.*, 2018; Morel *et al.*, 2014) and alcohol (Besson *et al.*, 2019), and to enhanced

659 appetite for food (Besson *et al.*, 2019), suggesting global alterations in reward processing. The  
660 apparently opposite influence of the  $\alpha 5$ SNP on cocaine-related behaviors, as compared to what  
661 was observed for other rewards, may rely on the specific inhibitory action of cocaine on nAChRs.

662  
663 The absence of effect of  $\alpha 5$  deletion on cue- and stress-induced reinstatement of cocaine seeking  
664 may be due to differences in the brain areas involved compared to cocaine-induced reinstatement  
665 (see (Kalivas and McFarland, 2003) and (Farrell *et al.*, 2018) for reviews). For example, the  
666 basolateral amygdala appears critical for cue-induced but not for cocaine- or stress-induced  
667 reinstatements (Kalivas and McFarland, 2003). The VTA, dorsal prefrontal cortex and NAcc  
668 shell are crucial to cocaine-induced reinstatement while the central nucleus of the amygdala and  
669 bed nucleus of the stria terminalis are necessary for stress-induced reinstatement (Kalivas and  
670 McFarland, 2003). In this line,  $\alpha 5$  is expressed at relatively high levels in the VTA and NAcc, but  
671 absent in the amygdala (Wada *et al.*, 1990). The lack of effect of *Chrna5* deletion on cue- and  
672 stress-induced relapse seems in accordance with the physiological absence of  $\alpha 5$  containing  
673 nAChRs in brain areas specifically involved in these modalities of relapse.

674

#### 675 ***1.4.3 Genetic association study in patients with CUD***

676 Attempting to translate these novel preclinical insights in a well-characterized human sample of  
677 patients with CUD, we first observed a lack of associations between SNPs from the cluster and  
678 CUD compared to controls, in contrast with two previous candidate gene association studies in  
679 humans reporting a protective effect of  $\alpha 5$ -SNP on cocaine dependence (Grucza *et al.*, 2008;  
680 Sherva *et al.*, 2010). This discrepancy may be explained by the different populations used as  
681 CUD cases. Thus, in our clinical sample, other biological and environmental risk factors for CUD

682 and pleiotropy between nAChRs genes and several SUDs may blur possible associations between  
683 *CHRNA5-B4-A3* genes and CUD. Moreover, if the protective effect of the  $\alpha 5$ SNP acts at early  
684 stages of cocaine use, as discussed above, it is not surprising not to detect it in our patients under  
685 a case/control design, since they mainly suffer from highly severe and comorbid “end-stage”  
686 CUDs. Indeed, in the sample of CUD patients, we observed a slower transition from 1<sup>st</sup> to  
687 disordered cocaine use in carriers of at least one copy of the  $\alpha 5$ SNP, which is in line with the  
688 preclinical experiment, further supporting its action on an early phase of CUD to mediate its  
689 putative protective effect. However, before testing  $\alpha 5$ -altering medication for CUD prevention,  
690 an extensive preclinical validation step would be required. Thus, although the prevention of the  
691 transition from ultra-high risk (UHR) states to full-syndrome disorders remains a major goal in  
692 psychiatry in general in order to reduce the burden of mental illness, it has yet been  
693 operationalized for schizophrenia only (McHugh *et al.*, 2018), with emerging evidence regarding  
694 CUD (Casey *et al.*, 2014). Selecting individuals with frequent cocaine use and homozygous for  
695 the  $\alpha 5$ -SNP genotype to provide them with preventive medication would require additional  
696 criteria, especially other biomarkers that remain to be identified. With that regard, a study  
697 recently identified addiction-like striatal activation patterns in self-reported cocaine users,  
698 representing a putative biomarker for CUD in people who did not fulfill CUD criteria (Cox *et al.*,  
699 2017). Eventually, combined with  $\alpha 5$ -SNP genotyping, this could open new avenues toward  
700 preventive clinical trials, while also probably raising ethical issues since such individuals would  
701 not be clinically ill.

702 Focusing on SNPs that alter *CHRNA5* expression in the brain, we further identified a SNP in  
703 *CHRNA4*, rs950776, associated with a shorter delay to relapse to cocaine use. Our focus was  
704 driven by the preclinical results showing increased cocaine relapse strength in  $\alpha 5$ KO rats, the

705 equivalent mutations of which in humans rather alter gene expression than protein structure  
706 (unlike rs16969968). The rs950776 SNP was previously associated with nicotine and alcohol  
707 dependence (Hancock *et al.*, 2015; Joslyn *et al.*, 2008), but not with CUD. This SNP is a  
708 synonymous and frequent mutation that does not affect *CHRNA4* expression. It is associated with  
709 increased *CHRNA5* expression, with the third largest Normalized Effect Size (NES) among the  
710 six SNP that we tested with CUD relapse (see **Table 1**). SNPs showing a larger NES than  
711 rs950776 had negative values but were not statistically associated with the relapse phenotype.  
712 Interestingly, rs950776 shows many associations with DNA enzymes, transcription factors and  
713 chromatin states, supporting its role as a regulatory variant. Finally, to verify whether this finding  
714 was in line or against our initial hypothesis that a more severe relapse phenotype in  $\alpha 5$ -KO rats  
715 should correspond to genetic associations resulting in decreased *CHRNA5* expression in patients,  
716 and in line with recent developments in the polygenic nature of human phenotypes and disease  
717 (Cano-Gamez and Trynka, 2020), we had to describe the meaning of this association in the  
718 broader genetic context of the *CHRNA3-CHRNA4-CHRNA5* regulome (Barrie *et al.*, 2017).  
719 Using methylation and expression data from available literature and online databases, we first  
720 found that rs950776 had been associated with the cis-methylation of *CHRNA5* promoter region  
721 (Hancock *et al.*, 2015) in the human brain. Second, we took into account the functional data from  
722 SNPs in the cluster that are in high LD (*i.e.* “co-inherited”) with rs950776. Although rs950776  
723 alone is associated with increased *CHRNA5* expression (NES =0.63), it is thus in strong LD with  
724 61 other SNPs significantly associated with either increased or decreased *CHRNA5* expression  
725 (not genotyped in the current study, data from the GTEx portal database, **Table S4**); resulting in a  
726 “weighted” NES of -2.46 in the NAcc (**Table 2**; *1.2.1.5.11 section* for the “weighted” NES  
727 calculation). This suggests that rs950776 may eventually be associated with decreased *CHRNA5*

728 expression through complex haplotypic and methylation effects, without evidence of other  
729 functional impact in the cluster. The impact of this SNP on relapse to cocaine use may thus be  
730 due to reduced *CHRNA5* expression, paralleling the increased relapse observed in  $\alpha 5$ KO rats.  
731 Among the other five SNPs tested with CUD relapse, *CHRNA5* rs680244 & rs667282 and  
732 *CHRNA3* rs3743074 & rs6495308 are associated with decreased *CHRNA5* expression, yet  
733 without being associated with CUD relapse in our study (see **Table 1**). We believe this is  
734 explained by the fact that *CHRNA4* rs950776 is the only SNP we tested that enhances expression  
735 of the *CHRNA5* antisense RNA RP11-650L12.2, which is decreased by rs680244, rs3743074 and  
736 rs938682. This effect toward reduced *CHRNA5* expression at the post-transcriptional level is  
737 likely to overcome that of other SNPs associated with decreased *CHRNA5* expression.

738

#### 739 ***1.4.4 Conclusion***

740 We showed that deletion of the  $\alpha 5$  nAChR subunit had no effect on cocaine-SA or on its  
741 extinction, but induced a major increase in cocaine-induced reinstatement of cocaine seeking.  
742 This effect was associated with increased neuronal activation in the NAcc shell. We further  
743 highlighted that the  $\alpha 5$ SNP rs16969968 was associated with impaired cocaine intake pattern at  
744 early stages of cocaine exposure but not at later stages, as supported both by our preclinical and  
745 clinical data. Finally, we identified a SNP in *CHRNA4*, rs950776, associated with a shorter delay  
746 to relapse to cocaine use in a sample of CUD patients, which may result from a reduction in  
747 *CHRNA5* expression.

748 These results suggest that *CHRNA5* expression may represent a novel biomarker for an increased  
749 risk of relapse to cocaine use and that positive allosteric modulators of  $\alpha 5$ -containing nAChRs  
750 could represent a novel therapeutic strategy against relapse to CUD. Such relapse prevention  
751 strategy is at the core of treatment guidelines against SUDs in general in order to limit their

752 chronicization and reduce their consequences. Future clinical trials based on this mechanism are  
753 greatly expected since CUD remains a devastating disorder that still lacks effective  
754 psychopharmacological treatment (Kampman, 2019).

755

756

757

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759

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782

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## 1.7 Figure and Table Legends

### **Fig. 1. Cocaine self-administration in WT, $\alpha$ 5SNP and $\alpha$ 5KO rats.**

#### **(A) WT, $\alpha$ 5SNP and $\alpha$ 5KO rats show no difference in active lever presses during food training.**

The two-way ANOVA performed on the number of food-pellet self-administered revealed no significant effect of genotype ( $F_{2,30}=0.77$ ,  $p=0.47$ , NS; partial  $\eta^2=0.1$ ), but a significant effect of session ( $F_{3,90}=90.99$ ,  $p<0.0001$ ; partial  $\eta^2=0.75$ ); and no interaction ( $F_{6,90}=0.79$ ,  $p=0.58$ , NS; partial  $\eta^2=0.05$ ).

#### **(B) WT, $\alpha$ 5SNP and $\alpha$ 5KO rats show no difference in active lever presses during the acquisition of the cocaine-SA (0.2 mg/kg/infusion).**

The two-way ANOVA performed on the number of active lever presses during these 13 sessions indicated a significant effect of session ( $F_{12,360}=22.31$ ,  $p<0.0001$ ; partial  $\eta^2=0.42$ ), but not of genotype ( $F_{2,30}=0.63$ ,  $p=0.54$ , NS; partial  $\eta^2=0.047$ ) nor interaction ( $F_{24,360}=1.43$ ,  $p=0.089$ , NS; partial  $\eta^2=0.087$ ).

#### **(C) Alteration in the number of cocaine infusions during the acquisition of the cocaine-SA (0.2 mg/kg/infusion) in $\alpha$ 5SNP rats compared to WT and $\alpha$ 5KO rats.**

The two-way ANOVA performed on the number of cocaine infusions during these 13 sessions indicated a significant effect of session ( $F_{12,360}=2.386$ ,  $p=0.0057$ ; partial  $\eta^2=0.07$ ) and interaction ( $F_{24,360}=1.69$ ,  $p=0.024$ ; partial  $\eta^2=0.1$ ), but not of genotype ( $F_{2,30}=2.68$ ,  $p=0.085$ , NS; partial  $\eta^2=0.19$ ). The one-way ANOVA performed on each group indicated a significant effect of session for  $\alpha$ 5SNP rats ( $F_{12,108}=2.79$ ,  $p=0.0024^{**}$ ), but not for WT or  $\alpha$ 5KO rats ( $F_{12,132}=1.79$ ,  $p=0.14$ , NS; and  $F_{12,108}=2.79$ ,  $p=0.056$ , NS).

#### **(D) Reduction in the number of cocaine infusions between the mean of 3 sessions under FR-1 and FR-5 during the acquisition of the cocaine-SA (0.2 mg/kg/infusion) in $\alpha$ 5SNP rats but not WT or $\alpha$ 5KO rats.**

The two-way ANOVA performed on the number of cocaine infusions received between FR-1 and FR-5 sessions indicated an absence of effect of session ( $F_{1,30}=1.24$ ,  $p=0.27$ , NS; partial  $\eta^2=0.04$ ) or genotype ( $F_{2,30}=1.88$ ,  $p=0.175$ , NS; partial  $\eta^2=0.25$ ), but a significant interaction ( $F_{2,30}=4.6$ ,  $p=0.018$ ; partial  $\eta^2=0.23$ ). Post-hoc analysis (paired t-test) FR-1 vs FR-5: WT ( $p=0.35$ , NS);  $\alpha$ 5SNP ( $p=0.0136^*$ ) and  $\alpha$ 5KO ( $p=0.21$ , NS) on reinstatement (NS on extinction).

#### **(E) WT, $\alpha$ 5SNP and $\alpha$ 5KO rats show no difference in the number of cocaine infusions during cocaine (0.1-0.8 mg/kg/infusion)-SA under FR-5 schedule of reinforcement.**

The two-way ANOVA indicated a significant effect of cocaine concentration ( $F_{3,75}=53.88$ ,  $p<0.0001$ ; partial  $\eta^2=0.68$ ), but not of genotype ( $F_{2,25}=0.13$ ,  $p=0.88$ , NS; partial  $\eta^2=0.018$ ) nor interaction ( $F_{6,75}=0.45$ ,  $p=0.84$ , NS; partial  $\eta^2=0.03$ ).

#### **(F) WT, $\alpha$ 5SNP and $\alpha$ 5KO rats show no difference in the number of active lever presses during five consecutive sessions with saline substituted for cocaine and cocaine associated cue still present.**

The two-way ANOVA indicated a significant effect of session ( $F_{4,92}=51.46$ ,  $p<0.0001$ ; partial  $\eta^2=0.69$ ) but not of genotype ( $F_{2,23}=0.84$ ,  $p=0.44$ , NS; partial  $\eta^2=0.05$ ) nor of interaction ( $F_{8,92}=0.82$ ,  $p=0.58$ , NS; partial  $\eta^2=0.07$ ).

#### **(G) WT, $\alpha$ 5SNP and $\alpha$ 5KO rats show no difference in number of cocaine infusions under a progressive ratio of reinforcement schedule at the cocaine doses of 0.2 and 0.4 mg/kg/injection.**

The two-way ANOVA indicated no significant effect of genotype ( $F_{2,23}=0.57$ ,  $p=0.57$ , NS; partial  $\eta^2=0.29$ ), cocaine concentration ( $F_{1,23}=1.13$ ,  $p=0.3$ , NS; partial  $\eta^2=0.047$ ), or interaction ( $F_{2,23}=0.29$ ,  $p=0.75$ ; partial  $\eta^2=0.025$ ).

**(H) WT,  $\alpha$ 5SNP and  $\alpha$ 5KO rats show no difference in Extinction behavior when cocaine and cocaine-associated cues were withheld.**

The two-way ANOVA indicated a significant effect of session ( $F_{7,161}=26.1$ ,  $p<0.0001$ ; partial  $\eta^2=0.53$ ); but not of genotype ( $F_{2,23}=0.007$ ,  $p=0.99$ , NS; partial  $\eta^2=0.0004$ ) nor interaction ( $F_{14,161}=0.82$ ,  $p=0.64$ , NS; partial  $\eta^2=0.066$ ).

Mean and SEM are represented.

**Fig. 2. Reinstatement of cocaine seeking in WT,  $\alpha$ 5SNP and  $\alpha$ 5KO rats and impact of the  $\alpha$ 5 subunit on cocaine-induced inhibition of cholinergic currents on nicotinic receptors.**

**(A) WT,  $\alpha$ 5SNP and  $\alpha$ 5KO rats show no difference in Reinstatement of cocaine seeking (active lever presses) after extinction induced by visual cue.**

The two-way ANOVA performed on cue-induced reinstatement indicated a significant effect of reinstatement ( $F_{1,23}=67.5$ ,  $p<0.0001$ ; partial  $\eta^2=0.74$ ); but not of genotype ( $F_{2,23}=0.14$ ,  $p=0.87$ , NS; partial  $\eta^2=0.013$ ) nor interaction ( $F_{2,23}=0.14$ ,  $p=0.87$ , NS; partial  $\eta^2=0.012$ ). ###:  $p<0.0001$  on extinction vs reinstatement for all genotypes (Bonferroni's multiple comparison test).

**(B) WT,  $\alpha$ 5SNP and  $\alpha$ 5KO rats show no difference in reinstatement of cocaine seeking (active lever presses) after extinction induced by pharmacological stress (Yohimbine 0.125 mg/kg; IP).**

The two-way ANOVA performed on stress-induced reinstatement indicated a significant effect of reinstatement ( $F_{1,23}=22.3$ ,  $p<0.0001$ ; partial  $\eta^2=0.49$ ); but not of genotype ( $F_{2,23}=0.08$ ,  $p=0.92$ , NS; partial  $\eta^2=0.007$ ) nor interaction ( $F_{2,23}=0.137$ ,  $p=0.87$ , NS; partial  $\eta^2=0.012$ ). ###:  $p<0.0001$  on extinction vs reinstatement for all genotypes (Bonferroni's multiple comparison test).

**(C) Enhanced reinstatement of cocaine seeking (active lever presses) after extinction induced by cocaine administration (10 mg/kg; IP) in  $\alpha$ 5KO rats but not in  $\alpha$ 5SNP or WT rats.**

The two-way ANOVA performed on cocaine-induced reinstatement indicated a significant effect of reinstatement ( $F_{1,23}=24.35$ ,  $p<0.0001$ ; partial  $\eta^2=0.51$ ); of genotype ( $F_{2,23}=6.47$ ,  $p=0.006$ ; partial  $\eta^2=0.34$ ) and interaction ( $F_{2,23}=5.97$ ,  $p=0.0082$ ; partial  $\eta^2=0.34$ ). Post-hoc analysis (Bonferroni's multiple comparison test)  $\alpha$ 5KO vs  $\alpha$ 5SNP or WT:  $p<0.001$  (\*\*\*) on reinstatement (NS on extinction). ###:  $p<0.0001$  on extinction vs reinstatement for  $\alpha$ 5KO but not  $\alpha$ 5SNP or WT rats (NS).

**(D) Enhanced expression of the c-Fos protein (number of c-Fos positive cells/mm<sup>2</sup>) during cocaine-induced reinstatement of cocaine seeking (coc) in the nucleus accumbens shell of  $\alpha$ 5KO rats compared to WT rats.**

The two-way ANOVAs performed on the number of c-Fos positive cells/mm<sup>2</sup> indicated a significant effect of treatment in the prelimbic cortex (PreL,  $F_{1,18}=19.5$ ;  $p=0.0003$ ), infralimbic cortex (IL,  $F_{1,23}=22.33$ ;  $p=0.0002$ ), cingulate cortex (Cg,  $F_{1,18}=20.6$ ;  $p=0.0003$ ), shell and core of the nucleus accumbens (shell,  $F_{1,18}=27.78$ ;  $p<0.0001$  and core,  $F_{1,18}=35.75$ ;  $p<0.0001$ ), anterior (a) and posterior (p) ventral tegmental area (VTAA,  $F_{1,15}=8.1$ ;  $p=0.012$  and pVTA,  $F_{1,18}=17.2$ ;  $p=0.0006$ ), anterior (a) and posterior (p) dorsal striatum (DSa,  $F_{1,17}=31.4$ ,  $p<0.0001$ ; DSa,  $F_{1,17}=48.6$ ,  $p<0.0001$ ) and primary motor cortex (M1,  $F_{1,16}=29.22$ ,  $p<0.0001$ ). The genotype effects were only significant in the NAcc shell ( $F_{1,18}=4.68$ ,  $p=0.044$ ), Post-hoc analysis (Bonferroni's multiple comparison test) WT vs  $\alpha$ 5KO \*:  $p<0.05$  on reinstatement (NS on extinction with saline condition). Saline vs cocaine ###:  $p<0.0001$ ; #:  $p<0.01$ ; #:  $p<0.05$ .

Mean and SEM are represented.

**Fig. 3. Crude and adjusted associations between the delay to transition from 1<sup>st</sup> to disordered cocaine use and *CHRNA5* rs16969968 genotype; and between the delay to relapse of cocaine use disorder and *CHRNA4* rs950776 genotype.**

**(A) Increased duration of the transition from 1<sup>st</sup> cocaine use to cocaine use disorder in patients (n=351) with cocaine use disorder carrying at least one copy of the rs16969968 polymorphism (*CHRNA5*).** Survival curve, time is expressed in months. Corrected p-value results from log-rank test.

**(B) The time to transition from 1<sup>st</sup> to disordered cocaine use is associated with rs16969968 binary genotype and the presence of lifetime opiate use disorder but not with the preferred route for cocaine administration during the self-reported worst period or the number of DSM-IV criteria for cocaine use disorder (Full cox model).**

Hazard ratios were all proportional (lowest p-value =0.093). Model is also adjusted for gender, and patients' protocol of origin, none of which were significant and thus not displayed (lowest p-value =0.49 for gender). \*p <0.05, \*\*p <0.01.

**(C) Reduction in the delay to relapse of cocaine use disorder in patients (n=306) with cocaine use disorder carrying at least one copy of the rs950776 polymorphism (*CHRNA4*).** Survival curve, time is expressed in months. P-value results from the cox proportional hazards test without additional covariates **Survival curves**

**(D) The delay to relapse of cocaine use disorder is associated with rs960776 binary genotype, the presence of lifetime alcohol and sedative medication use disorders, and the number of DSM-IV criteria for cocaine use disorder (full cox model).**

Hazard ratios were all proportional (lowest p-value =0.093).

Position on chromosome 15	SNP (rsID)	Gene	A2	A1	<i>P</i> -value: cocaine use disorder vs. 2461 controls	<i>P</i> -value: association with relapse of cocaine use disorder in the 6 selected SNPs	Normalized effect size of SNPs tested with relapse on <i>CHRNA5</i> expression in the Nucleus Accumbens
78859610	rs190065944	<i>CHRNA5</i>	G	A	NA		
78863472	rs667282		A	G	0.999	0.5657	-0.52
78871288	rs680244		G	A	NA	0.1876	-0.84
78882925	rs16969968		G	A	0.999		
78888400	rs578776	<i>CHRNA3</i>	G	A	NA		
78896547	rs938682		A	G		0.5457	0.53
78898723	rs12914385		G	A			
78907656	rs6495308		A	G	0.999	0.5657	-0.5
78908032	rs8042374		A	G	NA		
78909480	rs3743074		A	G	0.999	0.4025	-0.82
78911181	rs8040868		A	G	0.999		
<b>78926018</b>	<b>rs950776</b>	<i>CHRNA4</i>	<b>A</b>	<b>G</b>	NA	<b>0.0066*</b>	<b>0.76</b>
78927589	rs12440298		A	C	0.998		
78930510	rs1316971		G	A	NA		
78946633	rs12594247		G	A			
78959037	rs12594550		G	C	0.999		
78960529	rs6495314		A	C	NA		
78963994	rs922691		A	G			
78964498	rs8032156		G	A			
78985317	rs28661610		G	A	0.998		
79006442	rs11072793		A	G	NA		

**Table 1: Summary statistics for associations between eight SNPs in the *CHRNA5-A3-B4* cluster on chromosome 15 (logistic regression, column 6) and between six SNPs from the cluster associated with changes in *CHRNA5* expression and delay to relapse of cocaine use disorder (Cox survival model, column 7). \* Significant after Bonferroni-correction for 21 tests. Normalized effect sizes are from the GTEx portal database (<https://gtexportal.org/>).**

<i>Brain region</i>	<i>NES weighted by LD for all SNPs in high LD with rs950776</i>
Brain - Cerebellar Hemisphere	-3.94
Brain - Substantia nigra	-3.67
Brain - Cortex	-3.39
Brain - Hippocampus	-3.31
Brain - Frontal Cortex (BA9)	-3.22
Brain - Putamen (basal ganglia)	-3.02
Brain - Anterior cingulate cortex (BA24)	-2.94
Brain - Caudate (basal ganglia)	-2.62
Brain - Hypothalamus	-2.48
Brain - Nucleus accumbens (basal ganglia)	-2.46
Brain - Cerebellum	-1.92
Brain - Spinal cord (cervical c-1)	-1.67
Brain - Amygdala	2.52

**Table 2: Normalized Effect Sizes (NES) in each brain region with significant *CHRNA5* quantitative trait loci according to the *GTEx* database (<https://gtexportal.org/home/gene/CHRNA5>) for 61 SNPs in linkage disequilibrium (LD,  $r^2 > 0.6$ ) with *CHRNA4* rs950776.**