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Léa Tochon, Nadia Henkous, Morgane Besson, Uwe Maskos, Vincent David. Distinct Chrna5 mutations link excessive alcohol use to types I/II vulnerability profiles and IPN GABAergic neurons. *Translational Psychiatry*, 2024, 14 (1), pp.461. 10.1038/s41398-024-03164-8 . hal-04783611

**HAL Id: hal-04783611**

**<https://hal.science/hal-04783611v1>**

Submitted on 14 Nov 2024

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## ARTICLE OPEN



# Distinct *Chrna5* mutations link excessive alcohol use to types I/II vulnerability profiles and IPN GABAergic neurons

Léa Tochon<sup>1</sup>✉, Nadia Henkous<sup>1</sup>, Morgane Besson<sup>2</sup>, Uwe Maskos<sup>2</sup> and Vincent David<sup>1</sup>✉

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Genome wide association and animal studies have implicated genetic variations in *CHRNA5*, encoding the  $\alpha 5$  subunit-containing nicotinic acetylcholine receptors ( $\alpha 5^*$ nAChRs), as a risk factor for developing alcohol use disorders (AUDs). To understand how  $\alpha 5^*$ nAChR mutations may influence alcohol (EtOH) drinking behavior, we used a two-bottle choice procedure with intermittent access to alcohol in male and female transgenic mice expressing either the highly frequent human single nucleotide polymorphism ( $\alpha 5$ SNP/rs16969968) or a deletion of the *Chrna5* gene ( $\alpha 5$ KO). AUDs-related preconsummatory traits (anxiety, sensation-seeking and impulsivity) were assessed with a battery of relevant tasks (elevated-plus maze, novel place preference and step-down inhibitory avoidance). The implication of the  $\alpha 5$ -expressing IPN GABAergic neurons in AUDs and related behavioral traits was verified using neurospecific lentiviral (LV)-induced reexpression of the  $\alpha 5$  subunit in  $\alpha 5$ KOxGAD-Cre mice. Both  $\alpha 5$ SNP and  $\alpha 5$ KO mice showed over-consumption of EtOH, but displayed opposite vulnerability profiles consistent with Cloninger's subtypes of human AUDs.  $\alpha 5$ SNP mice showed Type I-like characteristics, i.e., high anxiety, novelty avoidance, whereas  $\alpha 5$ KOs exhibited Type II-like features such as low anxiety and high impulsivity. LV re-expression of the  $\alpha 5$  subunit in IPN GABAergic neurons restored the control of EtOH intake and improved the impulsive phenotype. We demonstrate that the SNP (rs16969968) or null mutation of *Chrna5* result in increased volitional EtOH consumption but opposite effects on anxiety, novelty-seeking and impulsive-like behaviors that match Cloninger type I and II of AUDs, including sex-related variations. IPN GABAergic neurons expressing  $\alpha 5^*$ nAChRs play a key role in limiting both EtOH drinking and motor impulsivity.

*Translational Psychiatry* (2024)14:461; <https://doi.org/10.1038/s41398-024-03164-8>

## INTRODUCTION

Alcohol use disorders (AUDs) are a world-wide problem and the third-leading risk factor for premature death and disability [1], with only three medications currently approved by the United States Food and Drug Administration [2]. Broad-ranging treatments show limited efficacy and are not applicable to all patients, thus development efforts have yet to take into account the heterogeneity of AUDs [3–5]. AUDs are multifactorial conditions with a strong heritable component, and genome wide association studies have linked many genetic alterations to AUDs [6]. Variants of the *CHRNA5* gene, which codes for the  $\alpha 5$  subunit of nicotinic acetylcholine receptors (nAChRs) are associated with high risk of schizophrenia, heavy smoking, but also AUDs [7–10]. For nicotine addiction, the strongest association was found with a missense mutation (rs16969968,  $\alpha 5$ SNP or D398N) for which the frequency worldwide is estimated at 28% and around 35% in Caucasians [11, 12]. An association of the  $\alpha 5$ SNP with symptoms of AUDs was also reported in individuals of European descent [13, 14], while others found no association [8, 15, 16]. Still, there is a strong correlation between smoking and alcohol abuse: an estimated 90% of alcohol users smoke, and alcohol dependence is 15 times more frequent in smokers than in non-smokers [17, 18]. A comprehensive review recently pointed out

the importance of investigating further the role of nAChRs and their therapeutic potential in AUDs [19].

The  $\alpha 5$ SNP changes codon 398 from GAT encoding Aspartate (D) to AAT encoding Asparagine (N) (D398N, or 397 in rodents), resulting in altered calcium permeability and concentration-response curves of  $\alpha 5$ -containing ( $\alpha 5^*$ ) nAChRs [20, 21]. In rodents,  $\alpha 5^*$ nAChRs are expressed in brain regions implicated in reward and aversion, i.e., the dopaminergic mesolimbic and habenulo-interpeduncular pathways, attention processes, impulsivity and memory (including the medial prefrontal cortex and hippocampus) [22, 23]. The highest expression of  $\alpha 5^*$ nAChRs is found in GABAergic neurons of the interpeduncular nucleus (IPN) [24, 25].

Similarly to human genetic studies, data on the implication of  $\alpha 5^*$ nAChRs in AUDs in rodents show mixed results. Rats carrying the  $\alpha 5$ SNP self-administered more alcohol (EtOH), required a higher dose of quinine to reduce their EtOH intake, and showed higher levels of reinstatement in response to EtOH and the cue together [26]. The loss of the  $\alpha 5$  subunit enhanced acute EtOH-induced effects resulting in lower tolerance, hypothermia and ataxia, hypnosis recovery time, and the anxiolytic-like response in mice, but no change in the metabolism of EtOH [26–28]. The deletion of *Chrna5* did not modify EtOH drinking in the Drinking-in-the-Dark paradigm, but it reduced EtOH-conditioned place

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Received: 2 October 2024 Revised: 8 October 2024 Accepted: 11 October 2024

Published online: 06 November 2024

preference and intake following restraint stress [27, 28]. Overexpression of  $\alpha 5^*$ nAChRs decreased EtOH self-administration without impacting the acute response to EtOH [29]. Interestingly, EtOH exposure elicited a persistent reduction of  $\alpha 5^*$ nAChRs in the posterior VTA of adolescent rats [30]. In human stem cells, exposure to low EtOH concentrations reduced *CHRNA5* expression during early development [31]. Overall, these data suggest that the  $\alpha 5$ SNP could increase the risk of AUDs, however its net effect and contributing mechanisms remain unclear.

Human and animal studies have provided converging evidence that specific personality traits, particularly anxiety, novelty seeking and impulsivity, are linked to AUDs [32, 33]. However, these traits are often measured after various degrees of EtOH use, leaving the question open on whether they confer vulnerability to AUDs or are induced by EtOH exposure. Therefore, it is critical to assess whether genetic mutations not only alter the pharmacological properties of EtOH but also the expression of specific personality traits. To better understand the impact of the  $\alpha 5$ SNP on AUDs we thus assessed anxiety, novelty-seeking, and impulsive behaviors before EtOH exposure in mutant mice either expressing the human  $\alpha 5$ SNP or null for *Chrna5* ( $\alpha 5$ KO) and WT mice. To verify the causal role of the  $\alpha 5$  subunit, we tested whether its reexpression in  $\alpha 5$ -enriched IPN GABAergic neurons was sufficient to modify AUDs-related and EtOH drinking behavior.

Males and females differ in their vulnerability to the reinforcing/addictive properties of drugs of abuse [34–36]. The  $\alpha 5$ SNP rs16969968 is associated with visual cue-induced neural reactivity in nicotine-dependent women [37]. Progesterone modulates the activity of  $\alpha 5^*$ nAChRs to alter anxiety-like behavior during estrus, and nicotine withdrawal increases IPN gene expression of  $\alpha 5$  in females in correlation with anxiety [38–40]. To date, however, only one study has tested male and female  $\alpha 5$ SNP mice, to conclude that the SNP increased EtOH drinking in the Drinking-In-the-Dark paradigm only in females [41]. It is therefore critical to conduct new experiments including male and female mice to better understand how sex and  $\alpha 5$  mutations interact.

## METHODS AND MATERIALS

### Animal models and care

All experimental procedures were approved by the Ethics Committee of the Bordeaux University (CEE50), the Animalerie Centrale, and Médecine du Travail, Institut Pasteur, the authors carrying out surgery hold an Animal Surgery and Project Authorization from the French Ministry of Agriculture (APAFIS#35136-2022061614593619). It is in accordance with the Guide for the Care and Use of Laboratory Animals (NIH) and the European Directive (2010/63/EU).

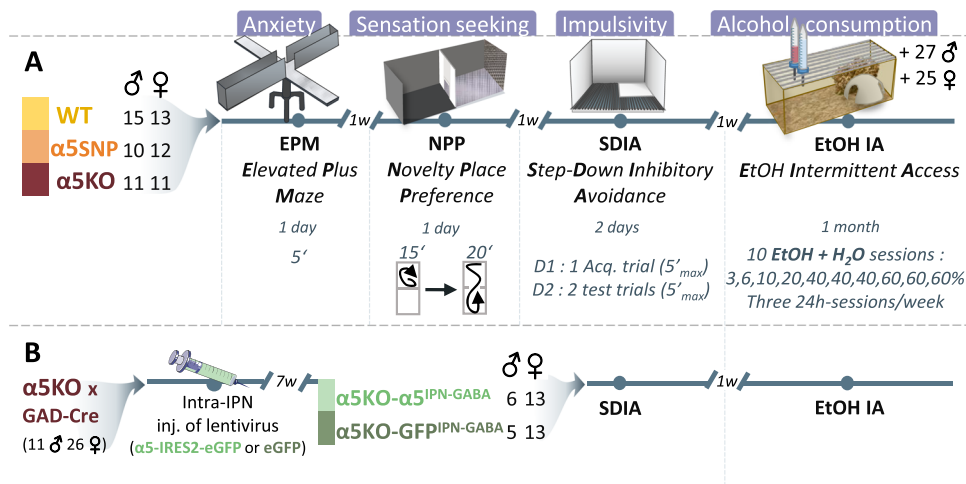
The study was conducted in male and female  $\alpha 5$ SNP transgenic mice, expressing the SNP (rs16969968) of the *Chrna5* gene encoding for the  $\alpha 5$ -nAChR subunit mutation D397N [42];  $\alpha 5$ KO mice with a constitutive deletion of *Chrna5*; and WT (C57BL6/J) littermate controls. We also used  $\alpha 5$ KOxGAD-Cre mice which received a Cre recombinase-activated lentiviral vector (LV) to drive the re-expression  $\alpha 5^{WT}$  specifically in IPN GABAergic neurons. WT,  $\alpha 5$ KO and  $\alpha 5$ SNP mice were littermates from cross-breeds of heterozygous ( $\alpha 5^{+/-}$ ) mice in our laboratory.  $\alpha 5$ KOxGAD-Cre mice were generated by the Pasteur Institute and housed in our facility for at least 1 month in the same room as WT,  $\alpha 5$ KO and  $\alpha 5$ SNP mice, matched for age and sex.

### Preconsumatory assessment of AUDs-related behavioral phenotypes

As described in Fig. 1,  $\alpha 5$ SNP and  $\alpha 5$ KO and WT littermate controls were first tested in a battery of three consecutive behavioral tasks to assess AUDs-related behavioral traits prior to alcohol exposure: [1] the elevated plus maze (EPM) [2] novelty place preference (NPP) and [3] step-down inhibitory avoidance task (SDIA). Following these three tasks we assessed voluntary alcohol drinking behavior in an EtOH intermittent access protocol [4] (EtOH IA) (Fig. 1A). We also determined blood alcohol level and basal plasmatic corticosterone levels. For detailed procedures of all behavioral tasks and biochemical assays see supplementary material.

### Intermittent access oral EtOH self-administration (two-bottle choice)

EtOH IA consisted in giving 24h-access to EtOH on alternating days with water available *ad libitum* as previously described [43–45]. A full description of the procedure is provided in the supplementary material.



**Fig. 1 Overview of experiments 1 and 2. A** Experiment 1: Effects of  $\alpha 5$  mutations on AUDs-related behavioral traits and alcohol drinking. Twenty-eight WT (15 ♂ 13 ♀), 22  $\alpha 5$ SNP (10 ♂ 12 ♀) and 22  $\alpha 5$ KO (11 ♂ 11 ♀) mice were tested in three consecutive behavioral tasks: (1) the elevated plus maze (EPM) (2) novelty place preference (NPP) and (3) step-down inhibitory avoidance task (SDIA); followed by (4) voluntary alcohol drinking behavior using a two-bottle choice intermittent access paradigm (EtOH-IA). Mice performed one behavioral task per week. Males and females were tested on separated days for the EPM, NPP and SDIA tasks but concurrently for the EtOH-IA. Fifty-two additional males and females of each genotype, not previously tested in the behavioral assessment battery, were included in the EtOH-IA to complete measures of the oral consumption. **B** Experiment 2: Effects of  $\alpha 5$  subunit re-expression in IPN GABAergic neurons on EtOH drinking and impulsivity. Thirty-seven  $\alpha 5$ KO x GAD-Cre (11 ♂ 26 ♀) mice received intra-IPN stereotaxic injections of lentivirus expressing either bicistronic  $\alpha 5$ -IRES2-eGFP or enhanced green fluorescent protein (eGFP) cDNAs to express, respectively, the  $\alpha 5$  subunit ( $\alpha 5$ KO- $\alpha 5^{IPN-GABA}$  mice) or GFP ( $\alpha 5$ KO-GFP<sup>IPN-GABA</sup> mice) in IPN GABAergic neurons. Seven weeks after stereotaxic surgery, 19  $\alpha 5$ KO- $\alpha 5^{IPN-GABA}$  (6 ♂ 13 ♀) and 18  $\alpha 5$ KO-GFP<sup>IPN-GABA</sup> (5 ♂ 13 ♀) were tested in the SDIA and EtOH-IA tasks to sex-matched WT,  $\alpha 5$ SNP and  $\alpha 5$ KO.

**Lentiviral re-expression of the  $\alpha 5$  subunit in IPN<sup>GABA</sup> neurons**  
 $\alpha 5$ KO x GAD-Cre ( $n = 37$ ) mice aged 12 to 24 weeks were anaesthetized using 250  $\mu$ l of ketamine (Merial, France)/xylazine 0.05% (Bayer Healthcare, France) in PBS and placed into a stereotaxic frame to receive bilaterally LV-expressing enhanced green fluorescent protein, from a PDGF-loxP-mCherry-loxP-GFP cassette ( $\alpha 5$ KO-GFP<sup>IPN-GABA</sup> mice,  $n = 18$ ) or bicistronic  $\alpha 5$ -IRES2-GFP from a PDGF-loxP-mCherry-loxP- $\alpha 5$ -IRES2-GFP cassette ( $\alpha 5$ KO- $\alpha 5$ <sup>IPN-GABA</sup> mice,  $n = 19$ ) into the IPN (antero-posterior:  $-3.60$  mm, lateral:  $\pm 1.60$  mm from sagittal line, and ventral:  $-4.50$  mm from Bregma and skull surface at an angle of  $15^\circ$ ). Vectors under the control of the PDGF promoter were used to generate viral particules in HEK-293T cells. The PDGF-loxP-mCherry-loxP-GFP was injected at 150 ng/2 $\mu$ l and the PDGF-loxP-mCherry-loxP- $\alpha 5$ -IRES2-GFP at 200 ng/2 $\mu$ l [42, 46]. For details about LV construction and histological controls see supplementary material.

### Statistical analysis

Statistical analyses were performed using two or three-way analyses of variance (ANOVA), with two between-subject factors: 'Genotype' (WT,  $\alpha 5$ KO,  $\alpha 5$ SNP) and 'Sex' (males vs females) and one within-subject factor with repeated measures ('Delay', 'Zone', 'Trial' or 'Dose'). To analyze the genotype effects specific to each sex, we performed two-way ANOVAs with one between-subject factor 'Genotype' (WT,  $\alpha 5$ KO,  $\alpha 5$ SNP) and one within-subject factor with repeated measures. Normal distribution, required for the use of ANOVA, was tested using the Shapiro-Wilk test. The variation within each group of data is reported as the standard error of the mean (SEM) and is included in all graphs. Post-hoc Bonferroni/Dunnett's multiple comparisons were performed following significant main effects. We also performed male and female comparisons using two-way ANOVAs with one between-subject factor ('Sex', males/females) and one within-subject factor with repeated measures. Paired  $t$ -test was used to compare the number of entries into the center vs corners in the NPP task. Simple linear regression analyses were performed with Blood EtOH Concentration (BEC) as dependent variable and Ethanol Intake (EI) as independent variable. All tests were considered statistically significant at  $p < 0.05$ .

## RESULTS

### AUDs-related behavioral phenotype and EtOH drinking in $\alpha 5$ SNP and $\alpha 5$ KO mice

**Anxiety-like behavior in the EPM.** As expected, WT mice showed a progressive increase in the time spent in open arms with no difference between males and females (Figs. 2A, and S1A). Compared to their WT littermates, null mutants displayed a hypo-anxious profile as assessed by increased open arms and extremities time, whereas  $\alpha 5$ SNP mice instead tended to be more anxious (Figs. 2A–C, and S1D). This inverted pattern between KO and SNP mice was more pronounced in males than in females.

**Novelty seeking behavior in the NPP task.** During the habituation phase (15 min-familiarization),  $\alpha 5$ KO males and females but not  $\alpha 5$ SNP (males or females) showed a WT-like response by crossing the center more often than corners (Fig. 2D). On the test day, WT and null mutants (males and females similarly) displayed a marked preference toward the novel compartment as revealed by positive novelty place preference (NPP) scores increasing over test time (Fig. 2E). In contrast,  $\alpha 5$ SNP males clearly avoided the novel compartment. There was no difference between  $\alpha 5$ KO,  $\alpha 5$ SNP and WT mice in their NPP scores or entry latencies in the novel compartment. Interestingly, however, the NPP score of  $\alpha 5$ SNP males decreased over time, thus revealing an avoidance of the novel compartment. Altogether, these results support the view that both male and female  $\alpha 5$ KO are prompted to explore novel spaces, whereas  $\alpha 5$ SNP males are quick to return to a familiar environment.

**Motor impulsivity and aversive learning in the SDIA task.** Combined increase in step-down latency and decreased escape latencies observed at 24 h and 26 h delays confirmed that all mice learned the task efficiently (Fig. 3A, B). There were no differences between  $\alpha 5$ KO,  $\alpha 5$ SNP and WT mice in step-down latency during the acquisition trial.

However, at 24 h and 26 h post acquisition,  $\alpha 5$ KOs tended to step down earlier than WT and  $\alpha 5$ SNP (Fig. 3A). Although this tendency was already visible in males,  $\alpha 5$ KO female mice displayed much shorter step-down latencies than sex-matched WT, leading to strong effects of Sex and Genotype (Fig. 3A right). Interestingly, there was no effect of Genotype on escape latency, but females took more time to get back on the platform during either 24 h or 26 h trials (Fig. 3B). During this task, we also recorded the freezing time expressed on the platform (before stepping down from it). Again, we observed an inverted pattern across  $\alpha 5$ KO and  $\alpha 5$ SNP mutants: the former displayed less freezing than WT or SNP mice, while in contrast the latter expressed more freezing than the two other groups (Fig. 3C).

**Alcohol drinking behavior in the EtOH-IA protocol.** WT males and females both increased their voluntary EtOH intake and preference over water dose-dependently (session 1 to 10, Fig. 3D). However, there was a leftward shift of the dose-effect curve in females as compared to males: WT females exhibited higher EtOH intakes and EtOH preference ratios than WT males for EtOH solutions ranging from 10 to 20% (Fig. 3D, E). The two  $\alpha 5$  mutations drastically increased voluntary EtOH consumption. Although  $\alpha 5$ KO and  $\alpha 5$ SNP mice did not differ from WT over sessions with 3, 6, 10 and 20% EtOH, both mutant strains showed higher EtOH intake and higher EtOH preference than WT during sessions with 40% EtOH in females, and up to 60% EtOH solutions in males (Fig. 3D, E). Therefore, effects of  $\alpha 5$  mutations also depend on sex in both  $\alpha 5$ KO and  $\alpha 5$ SNP mutants.

**Basal plasmatic corticosterone and blood EtOH concentrations.** Measures of basal plasmatic corticosterone revealed an inverted pattern in both nicotinic mutants, i.e., elevated in  $\alpha 5$ SNP but conversely reduced in  $\alpha 5$ KO mice as compared to WT littermates (Fig. 4A).

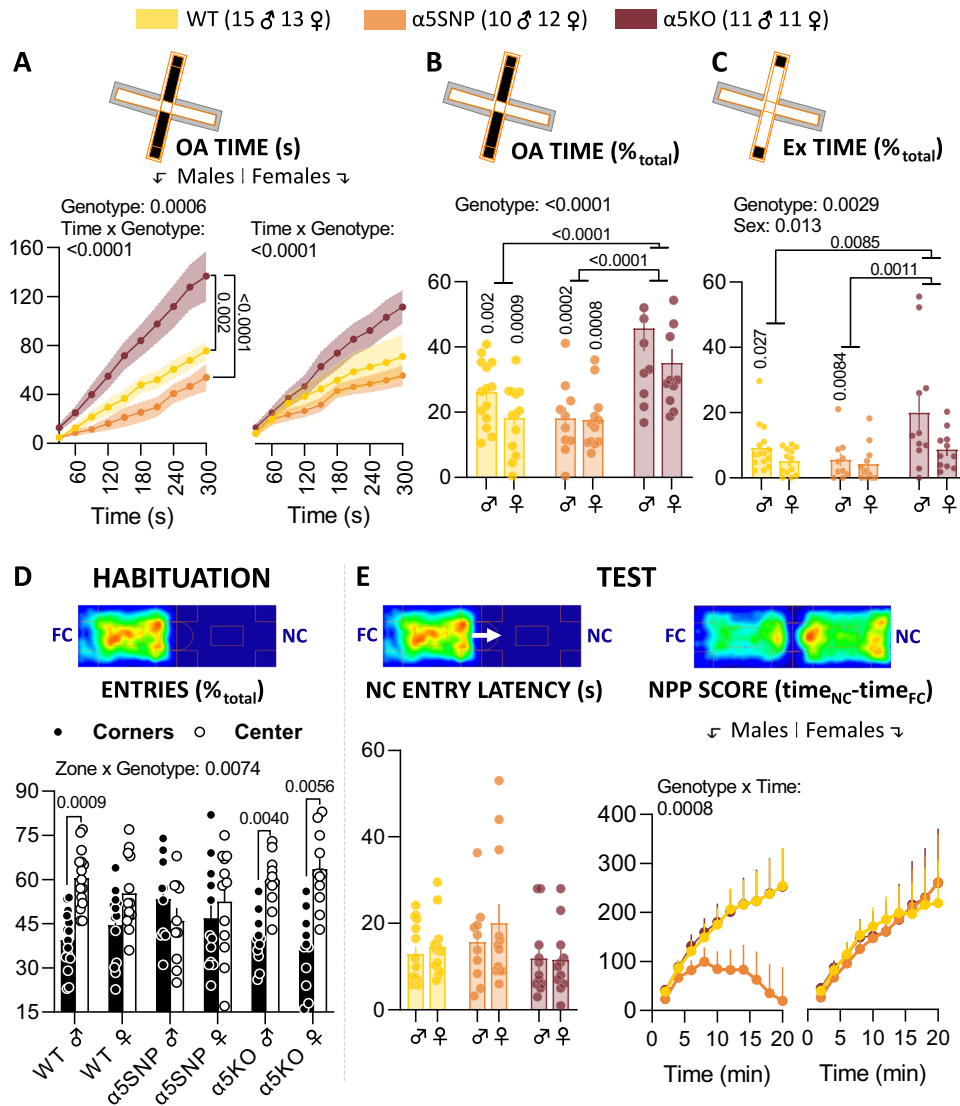
To exclude potential differences in alcohol metabolism among sexes and genotypes, we measured blood EtOH concentration after 30-min sessions with 40% and 60% EtOH. We found no influence of Genotype or Sex on blood EtOH concentration (Fig. 4B–F). As previously reported, the *Chrna5* genotype did not affect EtOH metabolism [26–28]. Therefore, we infer that changes in alcohol consumption in  $\alpha 5$  transgenic mice were not directly linked to *CHRNA5* mutations affecting EtOH metabolism. Likewise, discrepancies between males and females were not due to differences in EtOH metabolism.

### Re-expression of $\alpha 5$ in IPN GABAergic neurons, EtOH drinking and impulsivity

**Re-expression of the  $\alpha 5$  subunit in IPN GABAergic neurons reduced impulsive-like behavior.** Following LV-induced reexpression of the  $\alpha 5$  subunit in the IPN (Fig. 5A),  $\alpha 5$ KO-GFP<sup>IPN-GABA</sup>,  $\alpha 5$ KO-GFP<sup>IPN-GABA</sup> and WT mice were tested in the step-down inhibitory avoidance task to further evaluate the causal role of  $\alpha 5$ -expressing IPN GABAergic neurons in impulsive-like behavior and aversive learning. All mice learned the task efficiently as attested by trial-dependent increase of step-down latencies and decreased escape latencies (Fig. 5B, C). Importantly,  $\alpha 5$ KO and  $\alpha 5$ KO-GFP<sup>IPN-GABA</sup> females displayed shorter step-down latencies than their WT littermates during the 26h-delay trial, whereas  $\alpha 5$ KO- $\alpha 5$ <sup>IPN-GABA</sup> displayed a mean step-down latency that was similar to WT. The re-expression of  $\alpha 5$  improved impulsivity-like behavior of females, but this was not sufficient to restore a WT-like profile (Fig. 5C).

**Re-expression of the  $\alpha 5$  subunit in IPN GABAergic neurons decreased alcohol drinking.** Neurospecific re-expression of the  $\alpha 5$  subunit in IPN GABAergic neurons efficiently reduced EtOH consumption and preference in  $\alpha 5$ KO males and females over the 10 sessions (Fig. 5D, E). During the 40% EtOH sessions,  $\alpha 5$ KO and

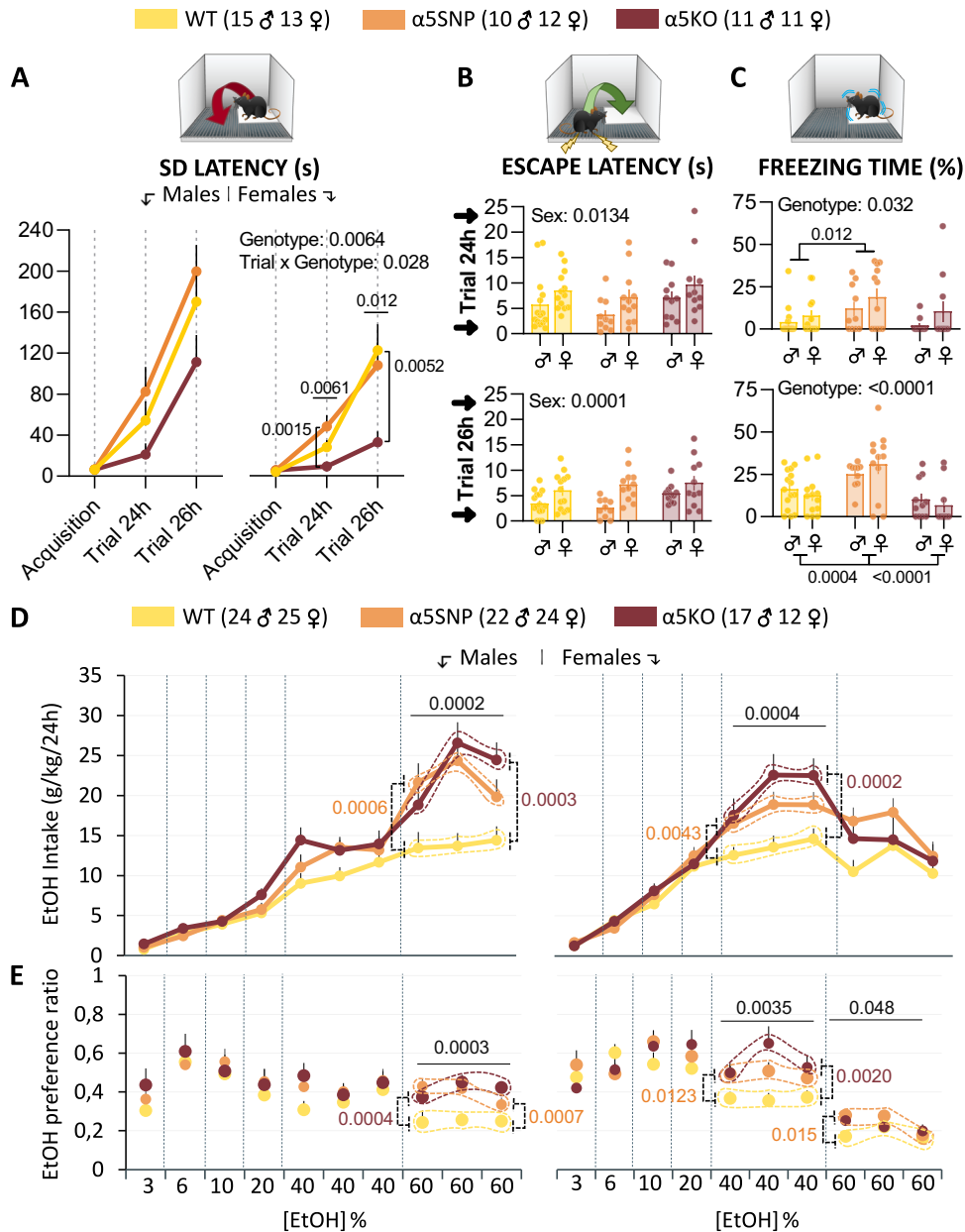




**Fig. 2 Anxiety-related behavior in α5SNP and α5KO mice.** Time spent on the EPM open arms (OA) per time segments (A) or on total test time (B) and in their extremities (Ext; C). There was a strong effect of Genotype on the three recorded parameters (OA time:  $F(2,66) = 10.25$ ,  $p = 0.0001$  and Time x Genotype interaction:  $F(18,594) = 10,952$ ,  $p < 0.0001$ ; %OA time:  $F(2,66) = 17.98$ ,  $p < 0.0001$ ; Ext time:  $F(2,66) = 6.39$ ,  $p = 0.0029$ ; A–C). α5KO spent more time, while conversely α5SNP mice tend to spend less time than WT in the OA ( $40 \pm 4\%$  vs  $18 \pm 2\%$  vs  $22 \pm 2\%$  respectively; α5KO vs WT:  $p < 0.0001$ , α5KO vs α5SNP:  $p < 0.0001$ ; cumulated in sec, α5KO vs WT:  $p = 0.0020$ , α5KO vs α5SNP:  $p < 0.0001$ ). Males and females α5KO showed an increase in OA time as compared to their sex-matched WT (Genotype effect, in males:  $F(2,33) = 9.37$ ,  $p = 0.0006$ ; Time x Genotype interactions, in males:  $F(18,297) = 7.99$ ,  $p < 0.0001$ ; in females:  $F(18,297) = 3.50$ ,  $p < 0.0001$ ), with the females having a more moderate response than males (Time x Sex interaction:  $F(9,594) = 2.99$ ,  $p = 0.0017$ ; A). The total OA time % was much higher in α5KOs than WT and α5SNPs in male ( $F(2,33) = 9.49$ ,  $p = 0.0006$ , α5KO vs WT:  $p = 0.0020$ , α5KO vs α5SNP:  $p = 0.0002$ ) and female mice as well ( $F(2,33) = 8.46$ ,  $p = 0.0008$ , α5KO vs WT:  $p = 0.0009$ , α5KO vs α5SNP:  $p = 0.0008$ ; B). A similar pattern was observed with “Ext” time parameter (C) in males but not in females showing instead a WT-like response (Ext % time:  $14 \pm 3\%$  vs  $7 \pm 1\%$  vs  $5 \pm 1\%$ ; α5KO vs WT:  $p = 0.0085$ , α5KO vs α5SNP:  $p = 0.0011$ ; in males:  $F(2,33) = 4.41$ ,  $p = 0.019$ , α5KO vs WT:  $p = 0.027$ , α5KO vs α5SNP:  $p = 0.0084$ ). Females spent less time in OA-Ext regardless of the genotype (Effect of Sex:  $F(1,66) = 6.45$ ,  $p = 0.013$ ). **Novelty seeking in α5SNP and α5KO mice.** Relative percentage of entries in the center versus corners of the habituation compartment (FC; D); entry latency in novel compartment (NC; E); and novelty place preference score during test trial (Test). There was a Zone x Genotype interaction during habituation ( $F(2,66) = 5.28$ ,  $p = 0.0074$ ). WT and α5KOs (males and females) crossed the FC center more often than corners (respectively:  $58 \pm 2$  vs  $41 \pm 2$ , paired  $t$ -test:  $p = 0.0005$ ; and  $62 \pm 2$  vs  $38 \pm 2$ , paired  $t$ -test:  $p < 0.0001$ ; D), but this effect was absent in α5SNP mice ( $50 \pm 3$  vs  $50 \pm 1$ ;  $p = 0.95$  ns). Similarly to WT males, α5KO males crossed the center more often than corners of the FC (α5KO:  $t(14) = 4.22$ ,  $p = 0.0040$ ; WT:  $t(10) = 3.70$ ,  $p = 0.0009$ ), while α5SNP males tended to do the opposite. In females, α5SNPs and WT entered the FC center as frequently as its corners (α5SNP:  $p = 0.60$  ns; WT:  $p = 0.12$  ns), while α5KOs crossed significantly more often the FC center than its corners ( $64 \pm 4$  vs  $36 \pm 4$ ;  $t(10) = 3.50$ ,  $p = 0.0056$ ). During the test there was an effect of Time ( $F(9,594) = 20.14$ ,  $p < 0.0001$ ) and a Genotype x Time interaction ( $F(18,297) = 2.49$ ,  $p = 0.0008$ , Test), due the avoidance of NC by α5SNP male mice.

α5KO-GFP<sup>IPN-GABA</sup> mice on the one hand, and α5KO-α5<sup>IPN-GABA</sup> and WT mice on the other hand, exhibited similar levels of consumption. Again, as previously observed, both parameters (EtOH intake and preference) decreased over 60% sessions in α5KO

females (Fig. 5D, E, right) while still increasing in males (Fig. 5D, E, left). Although α5KO-GFP<sup>IPN-GABA</sup> male mice did not reach 60% EtOH intake and preference as high as α5 null mutants, intake levels in α5KO-α5<sup>IPN-GABA</sup> mice were half of GFP controls (Fig. 5D, left).



## DISCUSSION

Although genome wide association studies have pointed toward a possible role of  $\alpha 5^*nAChRs$  in the risk of AUDs, preclinical studies have reported mixed results in support so far. Here we investigated how genetic variation of the *CHRNA5* gene could influence voluntary EtOH drinking using a two-step strategy. We first characterized (i) AUDs-related behavioral traits in male and female  $\alpha 5SNP$  and  $\alpha 5KO$  mice before EtOH exposure; and (ii) chronic volitional EtOH drinking behavior in an intermittent access protocol within a wide range of concentrations (3–60%).

We observed that both  $\alpha 5$  mutant strains exhibited a severe increase in volitional EtOH drinking as compared to their WT littermates. These results are consistent with the increase in relapse of EtOH self-administration previously reported in  $\alpha 5SNP$  rats [26], and the link between alcohol consumption and genetic variation in the *Chrna5* gene reported in mice [47]. Transgenic mice overexpressing the *CHRNA5/A3/B4* cluster instead

consumed less EtOH than WT [29]. Importantly, the present EtOH-IA model is a chronic paradigm running over four weeks, when 4 to 24h-access Drinking-In-the-Dark used in most animal studies is thought to be a model of binge drinking. Still, neither  $\alpha 5SNP$  nor  $\alpha 5KO$  differed from WT during sessions with 3 to 20% EtOH, which could account for the fact that no effect of *Chrna5* deletion on EtOH drinking was reported for EtOH doses below 20% [27, 28, 41, 48]. The maximum difference between either SNP or null mutant and WT peaked at concentrations as high as 40% in females and 60% in males regardless of the mutation. Neither the  $\alpha 5SNP$  nor deletion of the  $\alpha 5$  nAChR subunit affect saccharine consumption or taste reactivity to quinine in rodents [26, 27, 41], suggesting that observed differences in EtOH intake at high concentrations are not due to altered taste sensitivity. Collectively, our results thus reveal a *Chrna5* mutation and sex-dependent leftward shift in the dose-effect curve, likely due to a decreased sensitivity to the adverse/aversive effects of high EtOH doses in  $\alpha 5$  mutants.

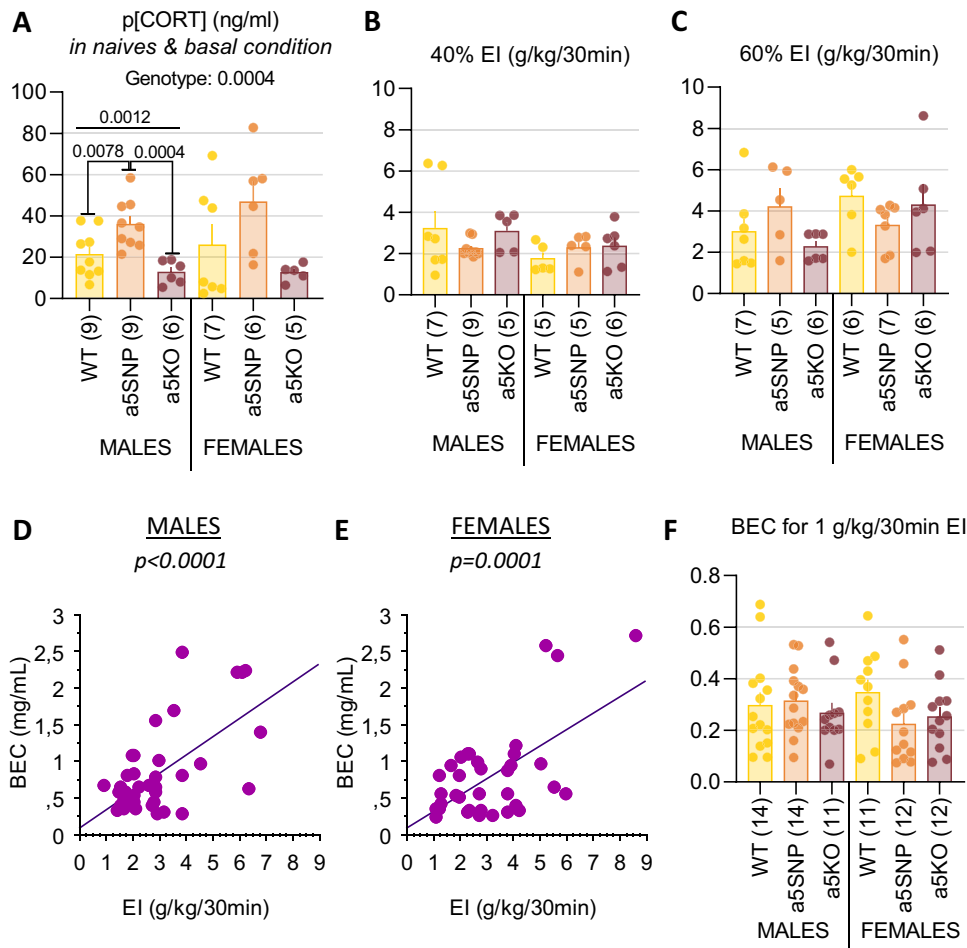
**Fig. 3 Impulsive-like behavior and alcohol drinking in  $\alpha 5$ SNP and  $\alpha 5$ KO mice. A** Step-down latency (SDL) during the acquisition, 24 h and 26h-delay retention trials. WT male and female mice learned the task efficiently (Trial effect in Males:  $F(2,66) = 70.22, p < 0.0001$ ; Females:  $F(2,66) = 37.88, p < 0.0001$ ).  $\alpha 5$ KOs exhibited shorter SDL than WT and  $\alpha 5$ SNP mice at either 24 h or 26h-delay trials (Genotype effect:  $F(2,66) = 7.35, p = 0.0013$ ,  $\alpha 5$ KO vs WT:  $p = 0.0050$ ,  $\alpha 5$ KO vs  $\alpha 5$ SNP:  $p = 0.0007$ ; Trial x Genotype interaction:  $F(4,132) = 5.01, p = 0.0009$ ). When analyzed by sex, this effect was significant only in females (Genotype effect, total:  $F(2,33) = 5.91, p = 0.0064$ ,  $\alpha 5$ KO vs WT:  $p = 0.0060$ ,  $\alpha 5$ KO vs  $\alpha 5$ SNP:  $p = 0.0043$ ; 24 h:  $F(2,33) = 5.98, p = 0.0061$ ,  $\alpha 5$ KO vs  $\alpha 5$ SNP:  $p = 0.0015$ ; 26 h:  $F(2,33) = 5.00, p = 0.012$ ,  $\alpha 5$ KO vs WT:  $p = 0.0052$ ) and was more pronounced over trials (Trial x Genotype interaction:  $F(4,66) = 4.51, p = 0.028$ ). All females were faster than males to step down (Sex effect:  $F(1,66) = 11.64, p = 0.0011$  and Trial x Sex interaction:  $F(2,132) = 9.24, p = 0.0002$ ). Female  $\alpha 5$ KO and  $\alpha 5$ SNP exhibited shorter SDL over trials than their male counterparts ( $\alpha 5$ KO:  $F(1,20) = 7.01, p = 0.015$ ;  $\alpha 5$ SNP:  $F(1,20) = 5.78, p = 0.026$ ). **B** Time to return on the platform (=escape latency: EL) for retention trials performed 24 h (**top**) and 26 h (**bottom**) after. This parameter decreased over trials in WT male and female mice (effect of Trial on EL, WT males:  $F(2,66) = 54.63, p < 0.0001$ ; WT females:  $F(2,66) = 64.36, p < 0.0001$ ). There was no effect of Genotype on EL at any delay, however female mice took generally more time to get back on the platform (effect of Sex on EL, 24 h:  $F(1,66) = 6.46, p = 0.0134$ ; 26 h:  $F(1,66) = 16.27, p = 0.0001$ ). **C** Fear-related behavior in the SDIA task as assessed by the freezing time expressed on the platform during trials at 24 h (**top**) and 26 h (**bottom**). The total freezing time was higher in  $\alpha 5$ SNPs than WTs (and  $\alpha 5$ KO) in either males ( $F(2,33) = 7.04, p = 0.0028$ ,  $\alpha 5$ SNP vs WT:  $p = 0.012$ ,  $\alpha 5$ SNP vs  $\alpha 5$ KO:  $p = 0.00080$ ) or females ( $F(2,33) = 6.54, p = 0.0040$ ,  $\alpha 5$ SNP vs WT:  $p = 0.0049$ ,  $\alpha 5$ SNP vs  $\alpha 5$ KO:  $p = 0.0028$ ). During the 24 h delay trial,  $\alpha 5$ SNPs displayed more freezing than WTs ( $F(2,66) = 3.61, p = 0.032$ ,  $\alpha 5$ SNP vs WT:  $p = 0.012$ ; **top**). During the 26 h delay trial, the SNP group displayed more freezing than WTs, whereas  $\alpha 5$ KOs expressed less freezing than their WT littermates ( $F(2,66) = 13.32, p < 0.0001$ ,  $\alpha 5$ SNP vs WT:  $p = 0.0004$ ;  $\alpha 5$ SNP vs  $\alpha 5$ KO:  $p < 0.0001$ ; **bottom**). **D–E** Dose-response curves for EtOH intake (EI in g/kg/24 h) and EtOH preference ratio over water (EP) in  $\alpha 5$ SNP,  $\alpha 5$ KO and WT mice. WT mice (males and females) increased their EI from  $1.04 \pm 0.1$  g/Kg/24 h to  $12.3 \pm 1.2$  g/Kg/24 h over sessions 3% to 60%-EtOH; and EtOH preference (EP) from  $0.20 \pm 0.02$  to  $0.39 \pm 0.05$ . Maximum EI was reached during 40%-EtOH in females and 60%-EtOH sessions in males (Sex x Dose interaction:  $F(9,1044) = 21.61, p < 0.0001$ ). EP was higher in females during sessions with 3–20%-EtOH (Sex x Dose interaction:  $p = 0.051$ ). Total fluid intake (EtOH + H<sub>2</sub>O) over 24h-period mice (3 ml) did not differ between males and females, but females being lighter than males ( $25 \pm 0.1$  g vs  $33 \pm 0.2$  g,  $F(1,116) = 146.67, p < 0.0001$ ) total fluid intake was higher in females when reported to the weight ( $2.6 \pm 0.05$  ml/20 g vs  $2.0 \pm 0.3$  ml/20 g,  $F(1,46) = 46.74, p < 0.001$ ). EtOH consumption was higher in both  $\alpha 5$ KO and  $\alpha 5$ SNP mice with a right-shift in EtOH doses from 40% in females to 60% in males (Genotype effect on EI:  $F(2,116) = 14.32, p < 0.0001$ ; and on EP:  $F(2,116) = 8.53, p = 0.0003$ ).  $\alpha 5$ KO and  $\alpha 5$ SNP males differed the most from WT males at concentration as high as 60%, with respective EI of  $23.3 \pm 1$  and  $21.9 \pm 1$  versus  $13.8 \pm 1$  g/kg/24 h in WTs (3 sessions at 60%:  $F(2,58) = 9.82, p = 0.0002$ ,  $\alpha 5$ KO vs WT:  $p = 0.0003$ ,  $\alpha 5$ SNP vs WT:  $p = 0.0006$ ; **3D left**) and respective 60%-EP ratio of  $0.42 \pm 0.02$  and  $0.39 \pm 0.02$  versus  $0.25 \pm 0.02$  in WTs (3 sessions at 60%,  $F(2,58) = 9.33, p = 0.0003$ ,  $\alpha 5$ KO vs WT:  $p = 0.00040$ ,  $\alpha 5$ SNP vs WT:  $p = 0.00070$ ; **3E left**). Only  $\alpha 5$ SNP females differed from WTs at 60% (EP =  $0.25 \pm 0.02$  vs  $0.18 \pm 0.02$ ; Genotype effect:  $F(2,58) = 3.18, p = 0.048$ ;  $\alpha 5$ SNP vs WT:  $p = 0.015$ ,  $\alpha 5$ KO vs WT:  $p = 0.19$  ns; **3E right**). Both  $\alpha 5$ KO and  $\alpha 5$ SNP females differed the most from WT females at 40%, with respective EI of  $20.9 \pm 1$  and  $18.1 \pm 1$  versus  $13.6 \pm 1$  g/kg/24 h in WTs (3 sessions at 40%:  $F(2,58) = 8.83, p = 0.0004$ ,  $\alpha 5$ KO vs WT:  $p = 0.0002$ ,  $\alpha 5$ SNP vs WT:  $p = 0.0043$ ; **3D right**) and respective 40%-EP ratio of  $0.56 \pm 0.04$  and  $0.49 \pm 0.03$  versus  $0.34 \pm 0.02$  in WTs (3 sessions at 40%,  $F(2,58) = 6.23, p = 0.0035$ ,  $\alpha 5$ KO vs WT:  $p = 0.0020$ ,  $\alpha 5$ SNP vs WT:  $p = 0.0123$ ; **3E right**). No difference with WTs was found in term of total fluid intake ( $\alpha 5$ KO:  $2.4 \pm 0.05$ ,  $\alpha 5$ SNP:  $2.4 \pm 0.04$ , WT:  $2.3 \pm 0.03$  ml/20 g).

EtOH is a potent allosteric modulator of the nAChR [18, 19, 49–51]. It modulates nAChRs dose-dependently and subtype-specifically, potentiating  $\alpha 6$ - and inhibiting  $\alpha 7$ -containing receptors at low doses, while higher doses affect  $\alpha 2\beta 2$ ,  $\alpha 2\beta 4$ ,  $\alpha 4\beta 2$ ,  $\alpha 4\beta 4$ , and inhibit  $\alpha 3\beta 2$ , with minimal effect on  $\alpha 3\beta 4$ . These modulations may result from alterations in the activation-desensitization balance of the receptor caused by ACh or nicotine [52–54], or from indirect signaling pathways like PKA inhibition [54]. Of note, there is evidence from *in vivo* and *in vitro* studies that EtOH increase ACh release into the VTA, driving activation of DAergic neurons through distinct nAChR subtypes [18]. How the SNP rs16969968 influence EtOH's effects on its parent receptor is currently unknown. It is likely to vary substantially depending on the associated subunits and the neuronal population expressing them. This SNP results in a partial loss of function, reduced Ca<sup>2+</sup> flux and resting state functional connectivity [55, 56]. Further research is needed to better understand the molecular mechanisms of EtOH- $\alpha 5^*$ nAChR interactions, but the recent identification of a subpopulation of “super-responsive” ChRNA<sup>+</sup> neurons to ACh opens up a promising new avenue of research [57]. Although the exact nature of the interaction between EtOH and  $\alpha 5^*$ nAChRs has yet to be fully elucidated, the present study provides further evidence highlighting the importance of these receptors in mediating alcohol-related behaviors.

The impact of  $\alpha 5$  mutations we observed on self-administration behavior is not exclusive to alcohol, as similar effects have been reported for nicotine at high concentrations [46, 58, 59], opiates and cocaine in rodents [60, 61], thus aligning with human studies [15, 62, 63]. Interestingly, the  $\alpha 5$ SNP is associated also with increased relapse to palatable food seeking as attested by higher breaking point in  $\alpha 5$ SNP rats [26], but normal acquisition of food self-administration (FR1-FR5) suggesting that potential effects on food rewards are not related to non-specific deficits in operant

learning or locomotion. Human studies investigating its impact on eating behavior are currently lacking. Collectively, these findings suggest that the rs16969968 variant may contribute to the development of several reward-related disorders and their comorbidities. One limit of our study is that we did not test whether  $\alpha 5$  mutants also meet other criteria for addiction-like behavior, such as punishment-resistant EtOH drinking, preference over other reward options and withdrawal syndrome [64–66]. However, it was recently suggested that therapeutic interventions could be more effective if carried out earlier in the addiction process, at a stage corresponding to moderate AUDs, i.e., 2 to 5 criteria of the DSM-5 [67]. The dysregulated EtOH drinking as characterized here by both increased intake and preference could be more relevant to such a ‘preaddictive’ state [67].

The IPN is the brain region most highly enriched with  $\alpha 5^*$ nAChRs which are expressed in GABAergic neurons, particularly within the rostral subnucleus projecting to the mesopontine raphe and dorsal tegmental area [24, 25, 68]. There is converging evidence that  $\alpha 5^*$ nAChR-expressing IPN GABAergic neurons mediate aversive states that may arise from chronic drug use [23–25, 68–70]. Using a previously validated lentivirus [42, 46], we thus tested whether the re-expression of the  $\alpha 5$  subunit in IPN GABAergic neurons would restore WT-like levels of EtOH drinking or limit its overconsumption in ChRNA5 mutants. Both *in vivo* / *in vitro* electrophysiological and ca<sup>2+</sup> imaging controls have confirmed that the intracranial delivery of the PDGF-lox-mCherry-loxP- $\alpha 5$ -IRES2-GFP lentiviral vector (LV-WT $\alpha 5$ ) allows the re-expression of functional  $\alpha 5^*$ nAChRs in the mouse brain [42, 46]. As expected, the spontaneous spiking activity of  $\alpha 5$ nAChR-expressing neurons was decreased in  $\alpha 5$ SNP mice, but is fully restored in mice having received the LV-WT $\alpha 5$  [42]. Based on this functional efficacy, we performed histological detection of viral GFP to confirm the re-expression of WT $\alpha 5$  in



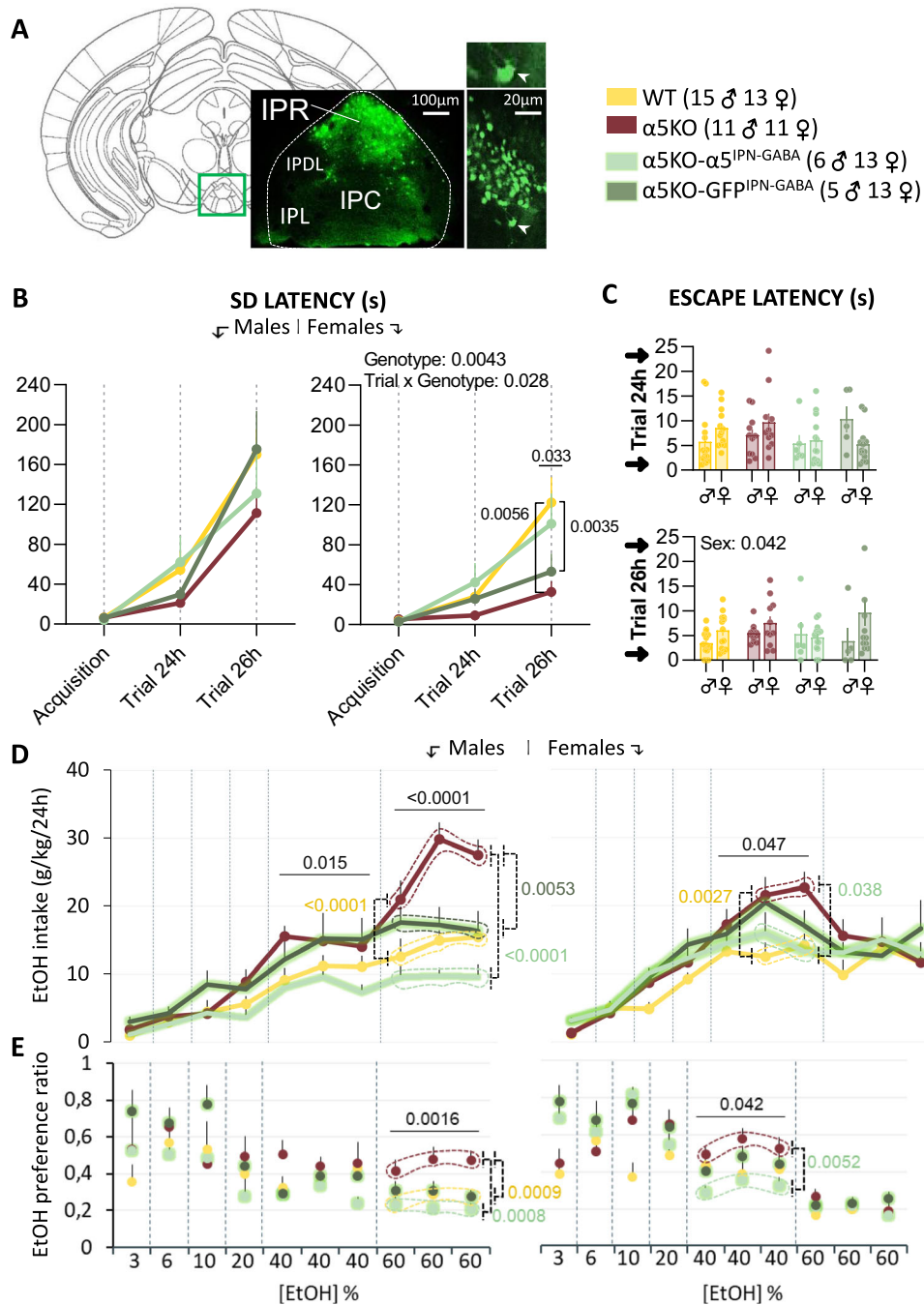
**Fig. 4** Basal plasmatic corticosterone and blood ethanol concentrations in  $\alpha 5$ SNP and  $\alpha 5$ KO mice. **A** WT mice exhibited similar pCORT in males and females (respectively:  $21.4 \pm 4$  ng/ml and  $25.9 \pm 10$  ng/ml),  $\alpha 5$ SNP showed higher pCORT levels ( $40.2 \pm 5$  ng/ml) than WT while conversely  $\alpha 5$ KOs exhibited lower pCORT levels ( $12.7 \pm 1$  ng/ml; Genotype effect:  $F(2,36) = 9.83$ ,  $p = 0.0004$ ,  $\alpha 5$ SNP vs WT:  $p = 0.0074$ ,  $\alpha 5$ KO vs WT:  $p = 0.10$  ns,  $\alpha 5$ SNP vs  $\alpha 5$ KO:  $p = 0.0002$ ). There was no effect of sex, but when analyzed separately the genotype effect reached statistical significance only in males ( $F(2,21) = 9.47$ ,  $p = 0.0012$ ,  $\alpha 5$ SNP vs WT:  $p = 0.0078$ ,  $\alpha 5$ KO vs WT:  $p = 0.13$  ns,  $\alpha 5$ SNP vs  $\alpha 5$ KO:  $p = 0.0004$ ; in females:  $p = 0.065$  ns). Blood EtOH concentration (BEC) induced by the EtOH-IA protocol as a function of EtOH data with 40% (**B**) and 60% EtOH solutions (**C**) in male and female mice for each genotype. After a 30 min-session, no difference was found between  $\alpha 5$  mutants and WT in terms of either 40%-EtOH/EP or 60%-EtOH/EP. BEC was positively correlated to their relative 40%-EtOH and 60%-EtOH in both male (**D**) ( $F(1,37) = 8.14$ ,  $p < 0.0001$ ) and female mice (**E**) ( $F(1,33) = 8.45$ ,  $p = 0.0001$ ). To make direct comparisons of BEC between mice, we divided the BEC (in mg/mL) of each mouse by their corresponding EtOH (g/Kg/30 min) to assess the BEC for 1 g/Kg/30 min EtOH (**F**). No Genotype or Sex difference was found with BEC for 1 g/Kg/30 min EtOH of:  $0.29 \pm 0.04$  (males) and  $0.34 \pm 0.05$  (females) in WT,  $0.26 \pm 0.04$  (males) and  $0.25 \pm 0.03$  (females) in  $\alpha 5$ KO and  $0.31 \pm 0.03$  (males) and  $0.22 \pm 0.04$  (females) in  $\alpha 5$ SNP (**F**).

GABAergic neurons of the targeted brain region (IPN). We found that LV-induced re-expression of the WT $\alpha 5$  in IPN GABAergic neurons decreased EtOH peak consumption for high concentrations in both male and female  $\alpha 5$ KOs. Moreover,  $\alpha 5$ KO- $\alpha 5^{\text{IPN-GABA}}$  consumed less EtOH than their GFP-injected counterparts. Together these results indicate that  $\alpha 5$ -expressing GABAergic neurons of the IPN may be a key element in the circuit regulating EtOH consumption, as previously demonstrated for nicotine [69, 70]. Notably,  $\alpha 5^*$ nAChRs are also expressed in the VTA where they act to limit nicotine self-administration [46]. However, since re-expression of  $\alpha 5$  in the IPN was Cre-directed specifically in GABAergic neurons, it is unlikely that its limiting effect on EtOH consumption is due to a direct action on VTA-DAergic neurons. In support, analysis of GFP labelling confirmed that re-expression was restricted to the IPN and targeted mostly the IPR subnucleus. An important open question, however, concerns the nature of IPN-related pathways involved in the control of EtOH intake. A recent study has provided new important evidence for direct reciprocal connections between

IPN and VTA [71]. Yet, the IPN is an anatomical hub projecting to various areas distributed throughout the brain and receiving many afferences [72]. Of note, 5-HT afferences from the Median Raphe Nucleus are also involved in the reward/aversion balance [73]. Future work should aim at identifying which IPN-connected structures mediate the limiting effects of  $\alpha 5^*$ nAChRs on volitional EtOH drinking.

The limiting effects of  $\alpha 5^*$ nAChRs on EtOH intake as demonstrated by the present study could be relevant to other drugs of abuse as well. Indeed, the  $\alpha 5$ SNP have been linked to changes in self-administration of nicotine [46, 58, 59, 74], morphine [60], and cocaine [61]. Interestingly, the reversal of increased nicotine self-administration due to  $\alpha 5$  knock-out was achieved by targeting  $\alpha 5$  re-expression in either MHb or VTA-DA neurons [46, 59]. One possibility is that this regulating effect results from the modulation of reward/aversion signaling. In support, the IPN is clearly involved in counteracting the activity of VTA-DA neurons [70, 75, 76]. However, other mechanisms could contribute to the control of drug use as well. For instance, we show that the LV-induced





re-expression of WTα5 also improved the level of impulsivity, and α5\*nAChRs are involved in anxiety and attention processes [42, 57], possibly reflecting an impact of the IPN α5 re-expression on targeted brain regions. Since these changes in behavioral traits can be detected before exposure, they may act as vulnerability endophenotypes predisposing to an excessive use of drugs to self-regulate emotional states or improve performance.

Another important observation of the present study is that, prior to any EtOH exposure, the two *Chrna5* mutations have profound effects on behavioral traits identified as factors of vulnerability to AUDs in humans and animals (anxiety, novelty seeking, impulsivity) [32, 33]. Moreover, personality traits can also influence treatment outcome. For instance, in US military veterans with polygenic risks for AUDs, positive psychosocial traits have a protecting effect against high alcohol consumption [77].

Therefore, it is crucial to better understand the impact of genetic variants, not only on the pharmacology of EtOH, but also on behavioral traits linked to excessive use.

We found that α5SNP mice displayed higher anxiety- and fear-related features in elevated plus maze, novelty place preference, and step-down inhibitory avoidance tasks, and higher basal corticosterone levels than WT littermates, thus revealing an increase in trait anxiety. In contrast, α5KOs exhibited hypoanxious responses and lower basal corticosterone levels. Consistently, the α5 subunit is highly expressed in the adrenal glands of humans and mice [78, 79], where it can influence plasmatic cortisol and corticosterone levels. Either no change [38] or increased anxiety [80] were sometimes observed in α5KO mice (only males). The sensitivity of anxiety to testing and housing conditions could account for these contrasting observations [81]. Present results are

### Fig. 5 Re-expression of the $\alpha 5$ subunit in GABAergic neurons of the IPN improves impulsive-like behavior and reduces alcohol drinking.

**A** Histological control of LV-induced re-expression of the  $\alpha 5$  subunit in the IPN. Analysis of all LV-injected  $\alpha 5\text{KO-GFP}^{\text{IPN-GABA}}$  and  $\alpha 5\text{KO-}\alpha 5^{\text{IPN-GABA}}$  mice included in this study ( $n = 37$ ) revealed that LV infection was restricted to IPN neurons. Strong eGFP signals were visualized in the rostral subnucleus of the IPN (IPR) known for expressing the highest density of GABAergic neurons which are highly enriched with native  $\alpha 5^* \text{nAChRs}$ . IPC: central subnucleus; IPL: lateral subnucleus; IPDL: dorsolateral subnucleus. **B** Step-down inhibitory avoidance task (SDIA). As previously observed (Fig. 3A), the step-down latency (SDL) increased over trials in  $\alpha 5\text{KO-GFP}^{\text{IPN-GABA}}$ ,  $\alpha 5\text{KO-GFP}^{\text{IPN-GABA}}$  and WT mice, yielding a Trial effect ( $F(2,158) = 82.28$ ,  $p < 0.0001$ ) and a Trial  $\times$  Genotype interaction ( $F(6,158) = 2.31$ ,  $p = 0.036$ ). In females, there was a strong Genotype effect on SDL at 26 h ( $F(3,46) = 3.16$ ,  $p = 0.033$ ) and  $\alpha 5\text{KO}$  and  $\alpha 5\text{KO-GFP}^{\text{IPN-GABA}}$  exhibited shorter SDL than their WT littermates ( $\alpha 5\text{KO vs WT: } F(1,22) = 9.42$ ,  $p = 0.0056$ ;  $\alpha 5\text{KO-GFP}^{\text{IPN-GABA vs WT: } F(1,24) = 4.98$ ,  $p = 0.035$ ). In contrast, female  $\alpha 5\text{KO-}\alpha 5^{\text{IPN-GABA}}$  mice displayed a WT-like phenotype (in sec:  $110.1 \pm 30$  and  $122.4 \pm 25$  in  $\alpha 5\text{KO-}\alpha 5^{\text{IPN-GABA}}$  and WT respectively; versus  $32.8 \pm 11$  and  $53.2 \pm 18$  in  $\alpha 5\text{KO}$  and  $\alpha 5\text{KO-GFP}^{\text{IPN-GABA}}$  respectively). **C** Evolution of escape latencies (EL) during SDIA retention trials at 24 h (top) and 26 h (bottom) after acquisition. EL decreased over trials:  $F(2,158) = 75.84$ ,  $p < 0.0001$ , with no difference between WT,  $\alpha 5\text{KO}$ ,  $\alpha 5\text{KO-GFP}^{\text{IPN-GABA}}$  and  $\alpha 5\text{KO-}\alpha 5^{\text{IPN-GABA}}$  mice during trials at 24 h or 26 h. **D** Dose-response curves for EtOH drinking assessed with EtOH intake (EI) and (E) preference (EP). Overall sessions and males/females combined,  $\alpha 5\text{KO}$  and  $\alpha 5\text{KO-GFP}^{\text{IPN-GABA}}$  ( $13.5 \pm 0.7$  and  $12.5 \pm 0.7$  g/kg/24 h) on one side and  $\alpha 5\text{KO-}\alpha 5^{\text{IPN-GABA}}$  and WT ( $9.2 \pm 0.4$  and  $9.9 \pm 0.5$  g/kg/24 h) on the other side showed similar levels of EI (Genotype effect:  $F(3,79) = 9.36$ ,  $p < 0.0001$ , WT vs  $\alpha 5\text{KO: } p < 0.0001$ , WT vs  $\alpha 5\text{KO-GFP}^{\text{IPN-GABA: } p = 0.0017$ ,  $\alpha 5\text{KO-}\alpha 5^{\text{IPN-GABA vs } \alpha 5\text{KO: } p = 0.0010$ ). In males (left),  $\alpha 5\text{KO-}\alpha 5^{\text{IPN-GABA}}$  mice showed lower levels of EI than  $\alpha 5\text{KO-GFP}^{\text{IPN-GABA}}$  and  $\alpha 5\text{KO}$  males overall sessions ( $F(3,33) = 14.50$ ,  $p < 0.0001$ ,  $\alpha 5\text{KO-}\alpha 5^{\text{IPN-GABA vs } \alpha 5\text{KO-GFP}^{\text{IPN-GABA: } p = 0.0022}$ ,  $\alpha 5\text{KO-}\alpha 5^{\text{IPN-GABA vs } \alpha 5\text{KO: } p < 0.0001}$ ,  $\alpha 5\text{KO vs WT: } p < 0.0001$ ). There was also a Genotype effect for 40%-EI ( $F(3,33) = 4.03$ ,  $p = 0.015$ ), 60%-EI ( $F(3,33) = 14.10$ ,  $p < 0.0001$ ,  $\alpha 5\text{KO-}\alpha 5^{\text{IPN-GABA vs } \alpha 5\text{KO: } p < 0.0001$ ,  $\alpha 5\text{KO-GFP}^{\text{IPN-GABA vs } \alpha 5\text{KO: } p = 0.0053}$ ,  $\alpha 5\text{KO vs WT: } p < 0.0001$ ), and 60%-EP as well ( $F(3,33) = 6.38$ ,  $p = 0.0016$ ,  $\alpha 5\text{KO-}\alpha 5^{\text{IPN-GABA vs } \alpha 5\text{KO: } p = 0.0008$ ,  $\alpha 5\text{KO vs WT: } p = 0.0009$ ). In females (right), there was a Genotype effect for both 40%-EI ( $F(3,46) = 2.86$ ,  $p = 0.047$ ) and 40%-EP ( $F(3,46) = 2.94$ ,  $p = 0.042$ ,  $\alpha 5\text{KO-}\alpha 5^{\text{IPN-GABA vs } \alpha 5\text{KO: } p = 0.0052$ ). Females  $\alpha 5\text{KO-}\alpha 5^{\text{IPN-GABA}}$  consumed less than  $\alpha 5\text{KO}$ s during session 2 and 3 of 40%-EI ( $\alpha 5\text{KO-}\alpha 5^{\text{IPN-GABA vs } \alpha 5\text{KO: } F(1,22) = 4.87$ ,  $p = 0.038$ ; WT vs  $\alpha 5\text{KO: } F(1,21) = 11.57$ ,  $p = 0.0027$ ).

in agreement with a previous report of hypoanxious-like behaviors in  $\alpha 5\text{KO}$  female mice [38]. Overall, we report converging, cross-task behavioral and physiological evidence for an opposite anxiety-related phenotype in  $\alpha 5\text{SNP}$  (hyper) and  $\alpha 5\text{KO}$  (hypo) mice. While showing opposite anxiety-related phenotypes, both  $\alpha 5$  mutants displayed excessive EtOH drinking, suggesting that either high or low emotional reactivity could equally act as a motivational drive underlying AUDs.

We also observed that  $\alpha 5\text{KO}$  displayed reduced inhibitory behavioral control (as measured with lower step-down latencies in the step-down inhibitory avoidance task). These results fit well with the high expression of  $\alpha 5^* \text{nAChRs}$  in the IPN and medial prefrontal cortex and their role in motor impulsivity and attentional processes [42, 82–84]. Consistently, transgenic mice over-expressing  $\alpha 3\alpha 5\beta 4^* \text{nAChRs}$  exhibit less impulsive-like behavior than WT controls, relative to the amount of copies of this transgene [85]. Finally, we show that LV-induced re-expression of WT  $\alpha 5$  improves the impulsive phenotype of  $\alpha 5\text{KO}$ , further supporting the role of  $\alpha 5^* \text{nAChR}$ -expressing GABAergic neurons of the IPN in this behavior.

The marked differences between  $\alpha 5\text{SNP}$  and  $\alpha 5\text{KO}$  mice across non-alcohol related behavioral tasks suggest that the partial loss of function of  $\alpha 5^* \text{nAChRs}$  resulting from the single polymorphism may have different consequences from the full knockout of these receptors in the functioning of specific IPN circuits [21, 55]. Particularly,  $\alpha 5$ -expressing GABAergic neurons projecting from the rostral IPN subnucleus to the median, paramedian, and dorsal raphe may be differently affected [69]. The raphe nuclei, central components of the brain serotonin system, produce and release serotonin, which regulates various physiological functions and whose deficiency is supposedly implicated in the pathophysiology of AUDs [86, 87]. Serotonergic hypofunction has been linked to early AUDs, impulsivity, and aggression, while hyperfunction may be anxiogenic and thus play a role in drinking as coping mechanism [88–91]. The behavioral patterns observed in  $\alpha 5\text{SNP}$  and KO mice resemble those associated with serotonergic hyper- and hypofunction, respectively. Hence, differential alterations in the IPN-raphe axis could account for the contrasting behavioral profiles between  $\alpha 5\text{SNP}$  and KO mice. In  $\alpha 5\text{SNP}$  mice, the mutated  $\alpha 5$  subunit might decrease neurotransmission along this pathway, whereas in  $\alpha 5\text{KO}$  mice, compensatory upregulation of neurotransmission could lead to contrasting behavioral outcomes.

Importantly, as for EtOH drinking, we observed sex-related variations or sex/mutation interactions in all behavioral traits assessed here. Females display more impulsive-like behavior and

this difference was most striking in KO mice. Female  $\alpha 5\text{SNP}$  mice lack novel place avoidance, observed in male  $\alpha 5\text{SNP}$ . Functional differences across the IPN of males and females could account at least in part for such effects. In rats, females, but not males, showed an increase in gene expression of  $\alpha 5$  following nicotine treatment or withdrawal, which correlated with anxiety, and levels of  $\alpha 5$  mRNA could be modulated by progesterone [38, 39]. Our study does not directly investigate the influence of estrous cycle phases on female behaviors. However, further investigation of the interplay between  $\alpha 5^* \text{nAChR}$  activity and estrous cyclicity could provide insights into the observed sex-related variations. These variations may vary in prominence depending on the specific hormonal phase of the female's estrous cycle. Independently from the hormonal factor, differences could also originate from structures closely linked to the IPN. A recent study already identified sex-dependent transcriptomic features in the habenula, that may underly opposite stereotyped parental behaviors displayed by female and male mice [92]. Therefore, in future investigations of IPN-related functions, it will be crucial to include male and female mice.

Collectively, our results thus show that while both nicotinic mutants are prone to excessive alcohol drinking,  $\alpha 5\text{SNP}$  and  $\alpha 5\text{KO}$  display clusters of behavioral traits (profile) that match remarkably with human AUDs subtypes initially established by Cloninger et coll. (1998) [93]. Type I is 'harm avoidant' and displays low scores on novelty seeking and high anxiety; Type II concerns a majority of males, with a 'sensation seeker' profile, low anxiety and high impulsivity. Many possible combinations can generate different motivations to consume EtOH, therefore various expressions of AUDs have long been suggested by clinicians. Binary, three-, four- and five-type models of alcohol dependence have been proposed [94]. Five alcoholism subtypes are recognized by The National Institute on Alcohol Abuse and Alcoholism (NIAAA): the young adult, functional Class, intermediate familiar, young antisocial and chronic severe subtypes [95]. Two subtypes proposed by Moss and coll. share similarities with the Cloninger's type I, while the three others resemble type II. Type I and II could represent the two extremes of a continuous spectrum that we might be able to model in rodents. Importantly, the Cloninger and Moss classifications come from community samples and not only from treatment-seeking AUDs patients further supporting the view that, consistently with present results, these vulnerability traits exist prior to AUDs.

CHRNA5 polymorphisms have also been linked to schizophrenia [9, 10, 96], often co-occurring with AUDs and nicotine dependence [97, 98]. Specifically, the  $\alpha 5\text{SNP}$  rs16969968 has been associated with elevated schizophrenia risk [10] and cognitive deficiencies

[96], along with heightened nicotine addiction severity in patients [99]. Cognitive impairment stands as a hallmark symptom of schizophrenia [100], accompanied by various other manifestations that may include increased anxiety [101], or greater impulsivity [102] further accentuated in individuals with comorbid lifetime AUDs [103]. Dysfunction in central  $\alpha 5^*nAChRs$  may underlie specific features of the schizophrenia spectrum [104]. In support, both  $\alpha 5$ SNP and  $\alpha 5$  knockout were associated with attentional deficits [42, 82, 105, 106]. We found that the  $\alpha 5$ SNP conferred higher anxiety, while the  $\alpha 5$ KO conferred an impulsive-like phenotype that may be improved by re-expression of WT  $\alpha 5$  in IPN neurons.  $\alpha 5^*nAChRs$  are strongly expressed in key brain structures implicated in schizophrenia, which further supports their involvement in this disorder (i.e., prefrontal cortex, ventral hippocampus, striatum, thalamus and MHB-IPN pathway) [107, 108]. In the prefrontal cortex, the  $\alpha 5$ SNP mice showed a major reduction in average activity of layers 2 and 3, mirroring the 'hypofrontality' observed in schizophrenic patients [42]. Rats with ventral hippocampal lesions in early life display memory and social deficits associated with schizophrenia, and also consume more alcohol [109]. Thereby, examining  $\alpha 5^*nAChR$  alterations in structures like the ventral hippocampus [24, 110] may offer new insights into schizophrenia and AUDs comorbidity that further complicates their management.

In conclusion, we provide evidence that alteration of  $\alpha 5^*nAChR$  function through a SNP variant (rs16969968) or *Chrna5* null mutation severely increases volitional alcohol consumption, and that  $\alpha 5^*nAChR$ -expressing GABAergic neurons in the IPN are implicated in limiting EtOH drinking and impulsivity-like behavior. These effects strongly depend on sex or sex/mutation interactions. The remarkable similarities between Type I/ $\alpha 5$ SNP and Type II/ $\alpha 5$ KO personality/behavioral traits suggest that these nicotinic mutants could work as a promising preclinical model to identify how AUDs subtypes differentially respond to existing behavioral, pharmacologic interventions or to evaluate new ones, for instance targeting  $\alpha 5^*nAChRs$ . This approach could accelerate the development of personalized, long-awaited more effective treatments.

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## ACKNOWLEDGEMENTS

This work was supported by the Centre National de la Recherche Scientifique CNRS UMR 5287 (V.D.) and CNRS UMR 3571 (U.M.), Bordeaux University, the Institut Pasteur, Agence Nationale de la Recherche (ANR), Neuroscience and BLANC, LABEX BRAIN, FP7 ERANET program NICO-GENE grant, French National Cancer Institute grant (INCa/CANCEROPOLE-TABAC-01-16022) (V.D, U.M), and FRM Equipe 2019 (U.M.). The laboratory of V.D. is part of the Bordeaux Neurocampus. U.M. is a member of the Laboratory of Excellence, Bio-Psy Labex; as such this work was supported by French state funds managed by the Agence Nationale pour la Recherche (ANR) within the Investissements d'Avenir program under reference ANR-11IDEX-0004-02. The team of U.M. is part of the Ecole des Neurosciences de Paris Ile-de-France Network.

## AUTHOR CONTRIBUTIONS

LT conceptualized the study, conducted behavioral experiments, analyzed the data, and drafted the initial version of the manuscript. NH designed, performed, and analyzed the biological assays, including plasmatic corticosterone and blood EtOH concentrations. MB assisted in the conceptualization, carried out neurospecific lentiviral reexpression, and reviewed the manuscript. UM assisted in the conceptualization, provided funding, supervised the lentiviral reexpression, and reviewed the manuscript. VD conceptualized the research, provided funding, supervised LT and NH, and reviewed the manuscript. All authors contributed to the article and approved the final submitted version.

## COMPETING INTERESTS

The authors declare no competing interests.

## ADDITIONAL INFORMATION

**Supplementary information** The online version contains supplementary material available at <https://doi.org/10.1038/s41398-024-03164-8>.

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