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Increased ethanol intake in prodynorphin knockout mice is associated to changes in opioid receptor function and dopamine transmission

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ABSTRACT

The purpose of this study was to examine the role of the prodynorphin gene in alcohol sensitivity, preference and vulnerability to alcohol consumption. Handling-induced convulsion (HIC) associated to alcohol, alcohol-induced loss of righting reflex (LORR), hypothermic effects in response to acute ethanol challenge, blood ethanol levels (BELs), conditioned place preference, voluntary ethanol consumption and preference, tyrosine hydroxylase (TH), dopamine transporter (DAT) and proenkephalin (PENK) gene expression, and μ-, δ- and κ-opioid agonist-stimulated [S^{35}]- guanosine 5′-triphosphate-binding autoradiography were studied in prodynorphin knockout (PDYN KO) and wild-type (WT) mice. There were no differences in HIC, LORR or the decrease in body temperature in response to acute ethanol challenge between PDYN KO and WT mice. PDYN KO mice presented higher BEL, higher ethanol-conditioned place preference and more ethanol consumption and preference in a two-bottle choice paradigm than WT mice. These findings were associated with lower TH and higher DAT gene expression in the ventral tegmental area and substantia nigra, and with lower PENK gene expression in the caudate–putamen (CPu), accumbens core (AcbC) and accumbens shell (AcbSh) in PDYN KO. The functional activity of the μ-opioid receptor was lower in the CPu, AcbC, AcbSh and cingulate cortex (Cg) of PDYN KO mice. In contrast, δ- and κ-opioid receptor-binding autoradiographies were increased in the CPu and Cg (δ), and in the CPu, AcbC and Cg (κ) of PDYN KO. These results suggest that deletion of the PDYN gene increased vulnerability for ethanol consumption by altering, at least in part, PENK, TH and DAT gene expression, and μ-, δ- and κ-opioid receptor functional activity in brain areas closely related to ethanol reinforcement.

Keywords Alcohol consumption, dopamine transporter, opioid receptor, prodynorphin gene, proenkephalin, tyrosine hydroxylase.

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INTRODUCTION

The opioid peptides regulate many behaviors, such as psychomotor stimulation, reward and positive reinforcement, alcohol and drug abuse consumption, schizophrenia, eating and sexual behaviors, mood, thermoregulation, pain and nociception (Emrich 1982; Mezey 1985; Schmauss & Emrich 1985; Henry 1986; Adler *et al.* 1988; Herz & Millan 1990; Furth, Wolterink & Ree 1995; Argiolas 1999; Ree *et al.* 2000; Shippenberg, LeFevour & Chefer 2008; Capasso, Putrella & Milano 2009). There are three major groups of endogenous opioid peptides (endorphins, enkephalins and dynorphins), which interact with at least three opioid receptor subtypes (μ, δ and κ) (Reisine & Bell 1993) with varied affinities and result in a wide variety of actions.

In the context of alcoholism, several studies have established that ethanol interacts with the opioid system to induce the release of opioid peptides, and modify the expression and function of opioid receptors. The main pathway that plays a critical role in the mediation of the reward and reinforcement processes of alcohol/drug abuse and dependence is the mesolimbic dopaminergic system (Wise 1987, 1998; Wise & Rompre 1989; Koob 1992a). This system sends dopaminergic projections from the ventral tegmental area (VTA; containing the body neuronal cells) to the ventral striatum, including the nucleus accumbens (NAcc) (Koob 1992a). In the

mesolimbic system, β-endorphin and enkephalin peptides, through their interactions with the μ- and δ-opioid receptors, increase dopamine (DA) release in the NAcc, which, in turn, produces rewarding or pleasurable effects and reinforces behavior (Iwamoto 1985; Shippenberg, Bals-Kubik & Herz 1987; Shippenberg & Herz 1987). In contrast, dynorphin peptides act on the κ-opioid receptors to reduce DA release, producing aversive and dysphoric states (Iwamoto 1985; Koob 1992a; Herz 1997, 1998; Chao & Nestler 2004).

Several studies have demonstrated that pharmacologic manipulation of opioid receptors may affect the reward properties of ethanol, and the acquisition and maintenance of ethanol consumption (Rubio, Ponce & Manzanares 2002b; Corchero, Manzanares & Fuentes 2004; Manzanares *et al.* 2005; Barrios de Tomasi & Juarez-Gonzalez 2007; Mendez & Morales-Mulia 2008; Soyka & Rosner 2008; Femenía *et al.* 2009; van Rijn, Brissett & Whistler 2010). For example, blockade of the central opioid receptors using selective and non-selective opioid antagonists may modulate the positive reinforcing properties of ethanol and be effective in reducing ethanol consumption. For instance, naltrexone, a μ-opioid receptor antagonist, decreases ethanol consumption in a number of animal species and experimental paradigms (Bienkowski, Kostowski & Koros 1999; Kamdar *et al.* 2007; Oliva & Manzanares 2007; Zalewska-Kaszubska *et al.* 2008; Czachowski & Delory 2009; Hill *et al.* 2010), and in patients with alcohol dependence (O'Malley *et al.* 1992; Rubio *et al.* 2002a; Kranzler, Wesson & Billot 2004; Garbutt *et al.* 2005; Ciraulo *et al.* 2008; Femenía *et al.* 2009; Garbutt 2010). On the other hand, intracerebroventricular infusion of opioid agonists, such as morphine, increases alcohol consumption in rats (Linseman & Harding 1990). These observations suggest an important role for μ-opioid receptors in the regulation of ethanol consumption. Indeed, mice lacking μ-opioid receptors do not self-administer ethanol in several paradigms (Roberts *et al.* 2000; Hall, Sora & Uhl 2001; Becker *et al.* 2002).

Blockade of the δ-opioid receptor reduces ethanol taste reactivity and ethanol consumption in outbred male rats, whereas activation of this receptor by intra-VTA microinjection of [D-Pen2,D-Pen5]enkephalin (DPDPE) decreases ethanol consumption in rats, particularly in low-drinking animals (Margolis *et al.* 2008). In addition, administration of the selective δ-opioid agonist TAN-67 reduces ethanol consumption in mice (van Rijn *et al.* 2010). In contrast, mice lacking δ-opioid receptors show greater preference for ethanol and consume more ethanol than their wild-type (WT) counterparts (Roberts *et al.* 2001). Moreover, deletion of the pre-proenkephalin (PENK) gene leads to a reduction in ethanol consumption (Blednov *et al.* 2004), while mutant mice with decreased β-endorphin expression show increased oral self-administration (Grisel *et al.* 1999).

On the other hand, the selective κ-opioid receptor agonist U-50,488H, given systemically, has been shown to reduce voluntary ethanol intake in rats (Lindholm *et al.* 2001) and to decrease cocaine or heroin self-administration (Glick *et al.* 1995; Kuzmin, Gerrits & Van Ree 1998; Xi, Fuller & Stein 1998; Schenk, Partridge & Shippenberg 1999). Furthermore, when κ-opioid agonists are administered systemically, they block both cocaine- and morphine-conditioned place preference (CPP) (Funada *et al.* 1993; Mori *et al.* 2002), induce dysphoria in humans (O'Brien, Volpicelli & Volpicelli 1996; O'Malley 1996), and cause conditioned place aversion in animals (Shippenberg & Herz 1986). In contrast, the blockade of κ-opioid receptors with the antagonist norbinaltorphimine increases ethanol consumption in rats (Mitchell, Liang & Fields 2005). However, Kovacs *et al.* (2005) found reduced ethanol consumption in mice lacking κ-opioid receptors.

Recently, Blednov *et al.* (2006), using prodynorphin knockout (PDYN KO) mice, proposed two possible and opposing roles for dynorphin peptides in the regulation of alcohol consumption. These authors suggested that peptides derived from PDYN may reduce the reinforcing actions of alcohol and that mice lacking these peptides should thus increase ethanol consumption. They further hypothesized that PDYN deletion may reduce the activation of κ-opioid receptors, resulting in less ethanol consumption (Blednov *et al.* 2006). However, these authors found decreased ethanol self-administration in PDYN KO females and no change in ethanol consumption in PDYN KO males compared with their respective WT littermates of the same sex. Since the stimulation of κ-opioid receptors decreases DA release, leading to aversive and dysphoric states (Iwamoto 1985; Herz 1998; Chao & Nestler 2004), it is plausible that dynorphin peptides act as a 'brake' to maintain the homeostatic balance of dopaminergic neuronal activity in the mesolimbic system. It is hypothesized that the removal of dynorphin peptides might impair this homeostatic control of DA release in the mesolimbic pathway, leading to increased vulnerability to ethanol consumption. The role that the dynorphin–κ-opioid system plays in ethanol consumption remains to be further clarified. In this study, ethanol consumption and preference, and responses to ethanol challenge [i.e. handling-induced convulsions (HIC), loss of the righting reflex (LORR) and hypothermic responses] in PDYN KO mice were examined. The expression of opioid receptors, opioid peptides, tyrosine hydroxylase (TH) and dopamine transporter (DAT) genes in key nuclei of the brain reward system in mice lacking the PDYN gene was also examined.

MATERIAL AND METHODS

Animals

Male pre-PDYN gene double mutant (PDYN KO) mice and WT C57BL/6 littermates were used in all the experiments. The generation of mice lacking the pre-PDYN gene has been described elsewhere (Sharifi *et al.* 2001). WT and PDYN KO mutant mice were matched for age and weight (age 2–3 months; weight 25–35 g). Mice were maintained at a constant room temperature of 23 ± 2°C and in a 12 hours/12 hours dark/light cycle (light from 8:00 AM to 8:00 PM), with free access to food and water. All the experiments were performed in compliance with the European Council Directive of 24 November 1986 (86/609/EEC).

Drug

Absolute ethanol (Sigma-Aldrich, Madrid, Spain) was diluted in saline for intraperitoneal (i.p.) administration (0.9% NaCl), or in water for *per os* (p.o.) administration.

Sensitivity to acute ethanol-induced hypothermia and sedation

Body temperature was determined in PDYN KO and WT mice ($n = 12$ mice/genotype) by inserting a rectal probe approximately 1 cm into the mouse's rectum (Radcliffe *et al.* 2005). Rectal temperature was measured at time 0 immediately before ethanol challenge (3 g/kg, p.o.) and 30, 60, 120, 240 and 480 minutes afterwards (3 g/kg, p.o.). The degree of hypothermia was expressed as the difference between the temperature at time zero (baseline) and the temperature recorded following the ethanol challenge.

The standard LORR time assay was used to evaluate ethanol sensitivity in PDYN KO and WT mice ($n = 10$ mice/genotype), as previously described (Crabbe *et al.* 2006; Nowak, Vinod & Hungund 2006; Vinod *et al.* 2008). A dose of 3.8 g/kg of ethanol was injected i.p. in PDYN KO and WT mice to induce the LORR. When mice became ataxic, they were placed in a V-shaped plastic piece in supine position. The LORR time was defined as the time from placement in the supine position until regaining the upright position in 30 seconds. As exclusion criteria for this test, we took into account the mice that failed to regain upright position within 30 seconds or had a LORR time greater than two standard deviations from the group mean.

Blood ethanol levels (BELs)

The plasma ethanol level was measured in PDYN KO and WT mice ($n = 10$ mice/genotype). Mice were sacrificed by decapitation 1 hour after receiving a dose of 3 g/kg of ethanol (p.o.). Trunk blood was collected into heparinized tubes to avoid blood coagulation. Ethanol concentration was determined by an enzymatic method, as described previously (Vinod *et al.* 2006). The blood was mixed with perchloric acid (90 µl of blood sample per 10 µl of perchloric acid) and the mixture was immediately vortexed and centrifuged. An aliquot of supernatant (50 µl) was incubated with reaction buffer [semicarbazide HCl buffer (67 mM); sodium pyrophosphate (67 mM) and glycine (20 mM) containing nicotinamide adenine dinucleotide (NAD) (1 mg) and alcohol dehydrogenase (0.5 mg)] for 1 hour at room temperature. The absorbance value was determined at $\lambda = 340$ nm.

HIC

Mice (PDYN KO and WT) were scored for HIC ($n = 8$ mice/genotype). A dose of 4 g/kg of ethanol was administered p.o. and the HIC score was measured immediately before mice were given ethanol and every hour after that until the HIC level reached baseline. Briefly, each mouse was picked up gently by the tail and, if necessary, gently rotated 180°. The HIC was scored as follows: 0, no convulsion; 1, facial grimace only after a gentle spin; 2, no convulsion when lifted, but tonic convulsion elicited by a gentle spin; 3, tonic–clonic convulsion after a gentle spin; 4, tonic convulsion when lifted; and 5, tonic–clonic convulsion when lifted (Crabbe *et al.* 1990; Crabbe, Merrill & Belknap 1991; Wilson & Little 1998; Farook *et al.* 2007).

Ethanol-conditioned place preference

Mice (PDYN KO and WT) were evaluated for their ethanol-CPP ($n = 10$ mice/genotype). The CPP apparatus consisted of two chambers ($30 \times 20 \times 20$ cm each) separated by a sliding door. One of the chambers was black and had a stainless steel grid rod floor; the other chamber was white and had a steel mesh floor. The walls of each chamber were decorated differently from the walls of the other chamber. The CPP procedure consisted of three phases, as follows (Cunningham, Ferree & Howard 2003): (1) pre-conditioning phase: day 1, mice were handled and brought to the conditioning room to minimize stress. Day 2, the animals were placed between the two chambers to allow free exploration of both chambers for a period of 20 minutes. Mice spending more than 70% of time in either of the two chambers were excluded from the experiment. (2) Conditioning phase: half of the animals of each genotype received water (p.o.) in the white chamber, while the other half received water in the black chamber on days 3, 5, 7 and 9. The same animals received ethanol [2 g/kg p.o., 20% (v/v) ethanol in water] in the opposite chamber on days 4, 6, 8 and 10. Animals remained in the conditioned chamber for 15 minutes. (3)

Test phase (day 11): the door was opened and mice were placed in the middle of two chambers to allow free exploration of the two compartments. The time that mice spend in each chamber was recorded for 15 minutes.

Voluntary ethanol consumption in a two bottle choice paradigm

Mice (PDYN KO and WT) were evaluated in a voluntary and chronic ethanol intake paradigm as previously described (Hungund *et al.* 2003). All mice were individually housed in small cages equipped with two tubes containing only water for acclimation during 1 week before the experiment. The two-bottle free choice paradigm with ethanol and water was as follows: one tube always contained water and the other contained gradually increased ethanol concentrations (2, 4, 6 and 8%, 4 days each). Food was available *ad libitum* and mice were weighed every 4 days. The volume of ethanol and water consumed were carefully measured every day with a specialized volumetric system that minimizes problems associated with leaks (Volumetric Drinking Tubes for Liquid Intake Monitoring—PHM-127A-15, Medical Associates Inc., St. Albans, VT, USA). The amount of ethanol consumed was calculated individually for each mouse and the values were averaged for each concentration of ethanol and expressed as grams per kilogram per day. The ratio of ethanol preference was also determined [volume of the ethanol solution consumed/volume of the total fluid consumed (i.e. ethanol + water)].

Histology

Coronal brain sections (12 μm) from brain regions of interest in the PDYN KO and WT mice ($n = 10$/genotype) were sliced using a cryostat. TH and DAT gene expression was studied in the substantia nigra (SN) and VTA. PENK gene expression and the functional activity of μ-, κ- and δ-opioid receptors were studied in the caudate–putamen (CPu), cingulate cortex (Cg) and NAcc core (AcbC) and shell (AcbSh). Brain coordinates were determined according to the Paxinos and Franklin Atlas (Paxinos & Franklin 2001).

In situ hybridization histochemistry (ISHH)

ISHH was performed as described previously (Young, Bonner & Brann 1986; Corchero, Fuentes & Manzanares 2002; Oliva *et al.* 2008), using a synthetic oligonucleotide probe complementary to PENK mRNA (bases 304–351, GenBank accession no. M13227; Amersham Pharmacia Biotech, Madrid, Spain), to TH mRNA (bases 1435–1482, GenBank accession no. M69200, Amersham Pharmacia Biotech) and to DAT mRNA (bases 1435–1482, GenBank accession no. M69200, Amersham Pharmacia Biotech). Oligonucleotide probes were labeled using terminal deoxytransferase (Amersham Pharmacia Biotech) to add a 35S-labeled deoxyadenosine triphosphate (1000 Ci mmol-1; Amersham Pharmacia Biotech) tail to the 3′ end. The probe (in 50 μl of hybridization buffer) was applied to each section and left overnight at 37°C for hybridization. Following hybridization, sections were washed four times for 15 minutes each in 0.15 M NaCl, 0.015 M sodium citrate, at pH 7.2 (1× saline sodium citrate, SSC) and 55°C, followed by two 30-minute washes in 1× SSC at room temperature, one brief water dip and blow-drying with air. To control for imaging enhancement variables, each set of slides was apposed to the same film (Kodak BioMax MR-1, Amersham Pharmacia Biotech) in individualized cassettes for 20 days (PENK) and 15 days (TH and DAT). Additional brain sections were co-hybridized with 100-fold excess of cold probe or with RNAse to assert the specificity of the signal. As expected, no hybridization signal was detected in these sections (data not shown).

Agonist-stimulated [S^{35}]-guanosine 5′-triphosphate (GTPγS) binding

Agonist-stimulated [S^{35}]GTPγS autoradiography was performed as previously described (Sim, Selley & Childers 1995). Coronal slides were rinsed in assay buffer (50 nM Tris, 3 mM MgCl2, 0.2 mM ethylene glycol tetraacetic acid, 100 mM NaCl, pH 7.4) at 25°C for 10 minutes and then incubated with 2 mM guanosine diphosphate (GDP) in assay buffer for 30 minutes at 25°C. Sections were then incubated for 2 hours at 25°C in assay buffer with 0.04 nM [S^{35}]GTPγS, 2 mM GDP and 3 μM, 7.5 μM or 3 μM of the opioid receptor agonists [D-Ala2, NMe-Phe4, Gly-ol5]-enkephalin (DAMGO) (μ), DPDPE (δ) or U50,448H (κ), respectively (Sigma-Aldrich). Baseline activity was evaluated in the absence of agonist. After incubation, slides were rinsed twice in 50 mM Tris buffer pH 7.4 and once in cold deionized water, air-dried and exposed to film for 24–48 hours.

Image analysis quantification

Autoradiograms were analysed with a computer using the public domain National Institutes of Health (NIH) Image program (developed at the US NIH and available on the Internet at http://rsb.info.nih.gov/nih-image). Optical densities were calculated from the uncalibrated mode by subtracting the corresponding background from each measurement and were expressed in grayscale values. The background measurement was taken from the area of the slice with the lowest non-specific hybridization signal and subtracted from the hybridization signal measurement in the same slice. In the autoradiograms from agonist-stimulated [S^{35}]GTPγS

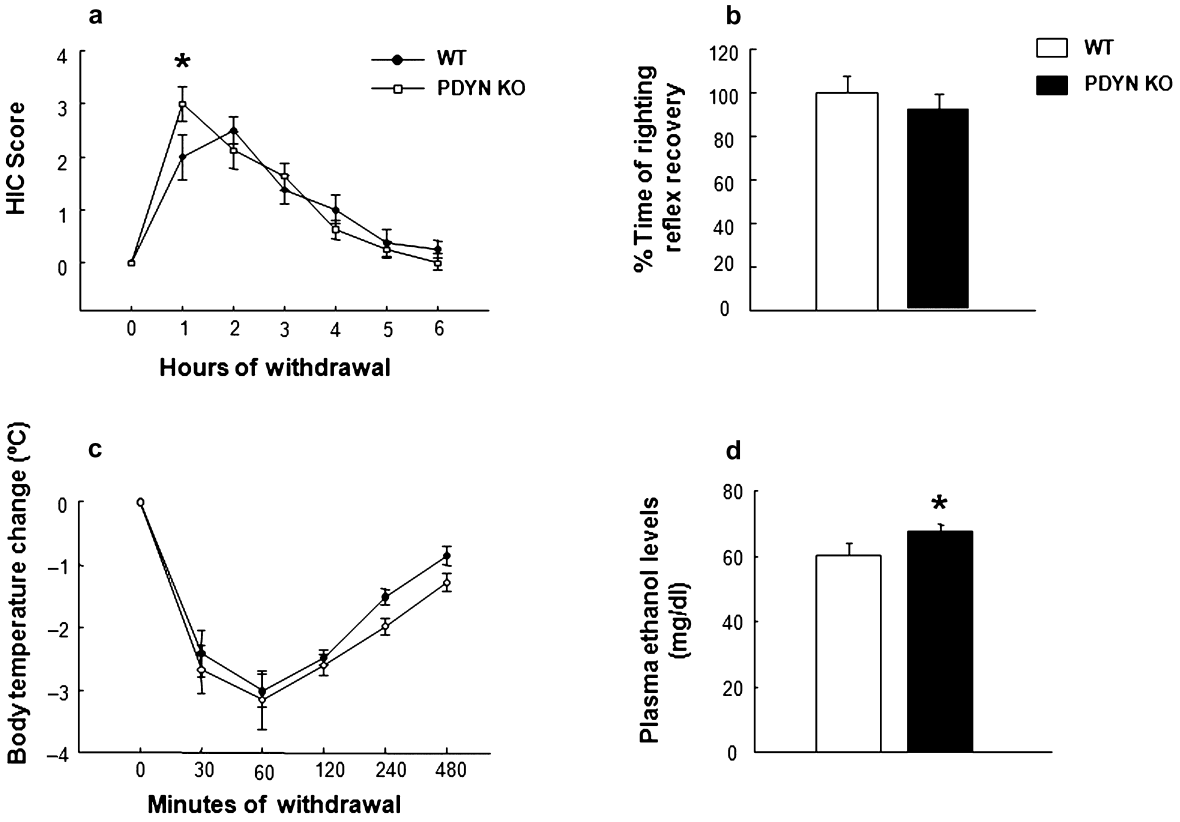

Figure 1 (a) Handling-induced convulsions (HIC) with ethanol-induced withdrawal (4 g/kg p.o.) in prodynorphin knockout (PDYN KO) and wild-type (WT) mice ($n = 8$). Symbols mark the means, and the vertical lines indicate the ±standard error of the mean (SEM) of HIC scores. *, Values from PDYN KO mice that differed significantly ($P < 0.05$) from WT mice. (b) Loss of righting reflex (3.8 g/kg i.p.) in PDYN KO and WT mice ($n = 10$). Columns represent the mean of time of righting reflex recovery expressed as a percentage of time. *, Values of time of righting reflex recovery from PDYN KO that differed significantly ($P < 0.05$, Student's *t*-test) from WT mice. (c) Ethanol-induced hypothermia (3 g/kg p.o., Student's *t*-test) in PDYN KO and WT mice ($n = 12$). Symbols represent the mean change in body temperature in degrees. *, Values from PDYN KO mice that differ significantly ($P < 0.05$) from WT mice. (d) Plasma ethanol level (3 g/kg p.o.) in PDYN KO and WT mice ($n = 10$). Columns represent mean plasma ethanol levels (mg/dl). *, Values from PDYN KO that differed significantly ($P < 0.05$, Student's *t*-test) from WT mice

autoradiography, optical densities were calculated by subtracting the corresponding 'baseline' value from each 'stimulated' measurement, as previously shown (Corchero *et al.* 1999). Results were shown considering mean control values as 100%.

Statistical analyses

Statistical analyses were performed with SigmaStat 3.1 software (Systat Software Inc., Chicago, IL, USA) using Student *t*-test when comparing values between two groups, two-way analysis of variance (ANOVA) followed by the Student Newman–Keul test when comparing two groups and two different factors, and two-way repeated measures (RM) ANOVA when comparing two groups and two different factors at repeated intervals. The level of statistical significance was $P < 0.05$. All values are expressed as the mean plus/minus standard error of the mean (SEM).

RESULTS

Sensitivity to acute ethanol-induced hypothermia, sedation, BEL and HIC

A single dose of ethanol (4 g/kg) produced more signs of withdrawal in PDYN KO mice compared with the control group in the first hour of withdrawal, a finding that was statistically significant ($P < 0.05$). The same dose of ethanol produced similar signs of withdrawal in both genotypes during the following 5 hours, until HIC baseline levels were regained, but this finding was not statistically significant ($P > 0.05$) (Fig. 1a). The time required by PDYN KO and WT mice placed in the supine position to regain the upright position was similar in both genotypes ($t = 0.743$; $P = 0.473$) (Fig. 1b); no mouse was excluded from the analysis because none differed considerably from the group.

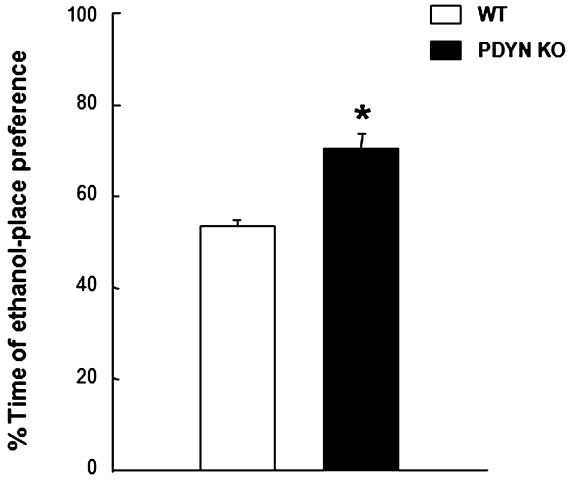

Figure 2 Conditioned place preference (CPP) for ethanol in prodynorphin knockout (PDYN KO) and wlld-type (WT) mice ($n = 10$). Columns represent the mean time spent in the place conditioned for ethanol in PDYN KO and WT mice, expressed as the percentage of total time. *, Values of time spent in the place conditioned for ethanol that differed significantly ($P < 0.05$, Student's *t*-test) from WT mice

After acute administration of ethanol (3 g/kg), PDYN KO and WT mice reached their greatest hypothermia at 60 minutes. The temperature in PDYN KO and WT mice decreased similarly at 30, 60 and 120 minutes and then gradually increased to reach almost baseline temperature at 480 minutes. There were no statistically significant differences between the two genotypes (two-way RM ANOVA {genotype [$F_{(1143)} = 1.64$; $P = 0.227$], time course [$F_{(5143)} = 43.26$; $P < 0.001$] and genotype × time course [$F_{(5143)} = 0.67$; $P = 0.649$]} (Fig. 1c).

The plasma ethanol level 1 hour after ethanol administration (3 mg/kg) was significantly higher (10%) in PDYN KO than in WT mice ($t = 3.97$; $P = 0.002$) (Fig. 1d).

Conditioned place preference

PDYN KO mice spent more time in the ethanol-paired chamber (19%, $P < 0.05$, Student's *t*-test) than WT mice (Fig. 2).

Voluntary ethanol consumption (two-bottle choice paradigm) and ethanol preference

PDYN KO mice showed increased voluntary ethanol consumption at 2, 4, 6 and 8% v/v of ethanol drinking compared with WT mice (Fig. 3). Two-way ANOVA revealed statistically significant differences between genotypes in the percentage of ethanol intake {genotype [$F_{(1319)} = 100.55$; $P < 0.001$], % ethanol [$F_{(3319)} = 19.89$; $P < 0.001$] and interaction genotype × % ethanol [$F_{(3319)} = 2.94$; $P = 0.033$]} (Fig. 3a). *Post hoc* analyses from two-way ANOVA ethanol consumption results, considering the ethanol concentration and differences between genotypes, revealed a statistically significant difference ($P < 0.005$) in ethanol consumption, which is represented by asterisks in Fig. 3a. Two-way RM ANOVA performed to analyse the progress in daily ethanol consumption showed that 'day' as a factor did not affect ethanol consumption in either genotype at 2% {day [$F_{(3,79)} = 1.683$; $P = 0.194$], genotype [$F_{(1,79)} = 17.41$; $P = 0.002$] and day × genotype interaction [$F_{(3,79)} = 0.847$; $P = 0.480$]}, 4% {day, [$F_{(3,79)} = 2.293$; $P = 0.101$], genotype [$F_{(1,79)} = 14.34$; $P = 0.004$] and day × genotype interaction [$F_{(3,79)} = 0.435$; $P = 0.730$]}, 6% {day [$F_{(3,79)} = 1.818$; $P = 0.168$], genotype [$F_{(1,79)} = 10$; $P = 0.012$] and day × genotype interaction [$F_{(3,79)} = 0.693$; $P = 0.564$]} and 8% {day [$F_{(3,79)} = 2.582$; $P = 0.074$], genotype [$F_{(1,79)} = 16.44$; $P = 0.003$] and day × genotype interaction [$F_{(3,79)} = 0.169$; $P = 0.916$]}. Moreover, *post hoc* tests indicated greater ethanol consumption in the PDYN KO mice compared with WT mice (Fig. 3b). In addition, ethanol preference was higher in PDYN KO mice compared with WT mice (Fig. 4). Two-way ANOVA showed statistically significant differences between genotype and percentage ethanol intake {genotype [$F_{(1319)} = 119.88$; $P < 0.001$], % ethanol [$F_{(3319)} = 3.12$; $P = 0.026$] and genotype × % ethanol interaction [$F_{(3319)} = 0.595$; $P = 0.619$]} (Fig. 4a). *Post hoc* analyses from two-way ANOVA to ethanol preference, considering the ethanol concentration and differences between genotypes, revealed a statistically significant difference ($P < 0.005$) in ethanol preference, which is represented by asterisks in Fig. 4a. Two-way RM ANOVA showed that 'day' as a factor did not affect ethanol consumption preference by both genotypes at 2% {day [$F_{(3,79)} = 0.325$; $P = 0.807$], genotype [$F_{(1,79)} = 19.48$; $P = 0.002$] and day × genotype interaction [$F_{(3,79)} = 1.383$; $P = 0.269$]}, 4% {day [$F_{(3,79)} = 0.935$; $P = 0.437$], genotype [$F_{(1,79)} = 14.83$; $P = 0.004$] and day × genotype interaction [$F_{(3,79)} = 0.803$; $P = 0.503$], 6% (day [$F_{(3,79)} = 0.893$; $P = 0.457$], genotype [$F_{(1,79)} = 44.11$; $P < 0.001$] and day × genotype interaction [$F_{(3,79)} = 0.093$; $P = 0.963$]} and 8% {day [$F_{(3,79)} = 0.407$; $P = 0.749$], genotype [$F_{(1,79)} = 69.54$; $P < 0.001$] and day × genotype interaction [$F_{(3,79)} = 0.440$; $P = 0.726$]}. *Post hoc* analyses from the two-way RM ANOVA between genotypes revealed a statistically significant differences ($P < 0.005$) in ethanol preference, represented by asterisks in Fig. 4b.

Gene expression studies

TH gene expression was significantly lower in PDYN KO compared with WT mice in the VTA (25.4%; $t = 2.62$;

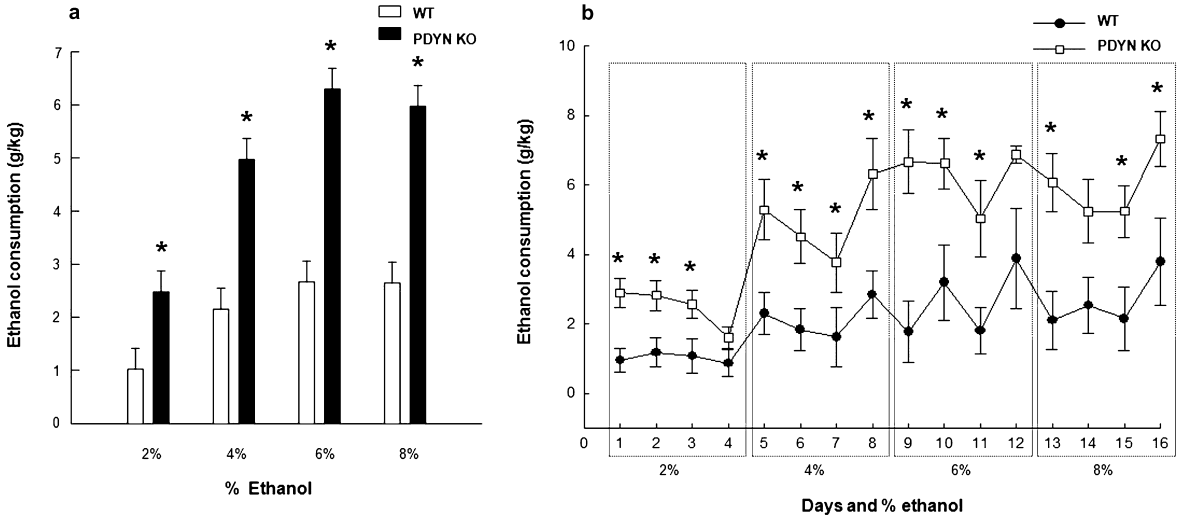

Figure 3 Voluntary ethanol consumption in prodynorphin knockout (PDYN KO) and wlld-type (WT) mice ($n = 10$). (a) represents the differences in ethanol consumption between the two genotypes at 2, 4, 6 and 8% v/v and (b) shows the progression of daily ethanol consumption by each genotype for a period of 16 days. The measures are volume of ethanol consumed every 24 hours, expressed as grams per kilogram per day. The ethanol concentration was gradually increased (2, 4, 6 and 8% v/v) every 4 days. Columns and symbols represent the mean ethanol consumption. *, Values from PDYN KO mice that differ significantly ($P < 0.05$) from WT

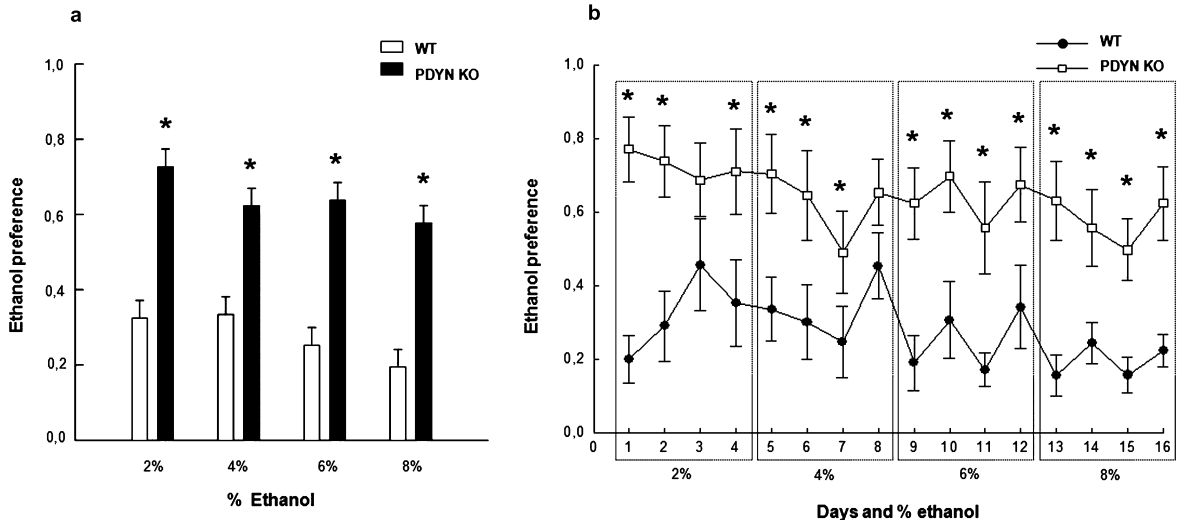

Figure 4 Preference for ethanol consumption in PDYN KO and WT mice ($n = 10$). (a) represents the ethanol preference in both genotypes at 2, 4, 6 and 8% v/v and (b) shows the progression of daily ethanol preference of each genotype for a period of 16 days. The measures were expressed as the ratio of preference for ethanol consumption (ethanol consumption/ethanol consumption + water consumption). Columns and symbols represent the mean ethanol preference. *, Values from PDYN KO mice that differed significantly ($P < 0.05$) from WT mice

$P = 0.024$) and SN pars compacta (SNc; 22.2%; $t = 2.48$; $P = 0.029$) (Fig. 5). However, DAT gene expression was significantly higher in PDYN KO compared with WT mice in the VTA (36%; $t = 4.2$; $P < 0.001$) and SNc (39%; $t = 4.86$; $P < 0.001$) (Fig. 6). PENK gene expression was significantly lower in PDYN KO compared with WT mice in the CPu (22.82%; $t = 3.082$; $P = 0.008$), AcbC (28%; $t = 4.063$; $P = 0.001$) and AcbSh (34.44%; $t = 3.83$; $P = 0.002$) (Fig. 7).

Agonist-stimulated [S^{35}]-GTPγS binding autoradiography of opioid receptors

DAMGO-stimulated [S^{35}]-GTPγS binding, an index of the functional activity of the μ-opioid receptor, was significantly lower in PDYN KO compared with WT mice in the CPu (27%; $t = 2.98$; $P = 0.008$), AcbC (20.7%; $t = 1.95$; $P = 0.067$), AcbSh (13.4%; $t = 1.092$; $P = 0.291$) and Cg (43.7%; $t = 3.10$; $P = 0.006$) (Fig. 8). Study of the

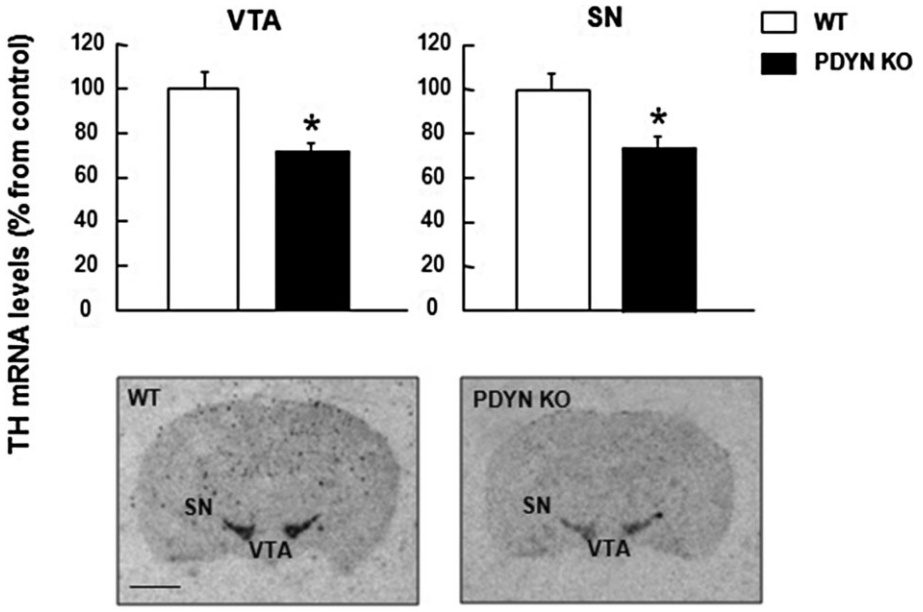

Figure 5 Tyrosine hydroxylase (TH) gene expression in the ventral tegmental area (VTA) and substantia nigra (SN) of PDYN KO and WT mice ($n = 10$). Columns represent the percentage of mean values of arbitrary units of optical density. *, Values of TH mRNA levels that differed significantly ($P < 0.05$, Student *t*-test) from WT mice. The lower panels show the representative autoradiograms of coronal brain sections at the level of the VTA and SN. (Bar represents 1 mm.)

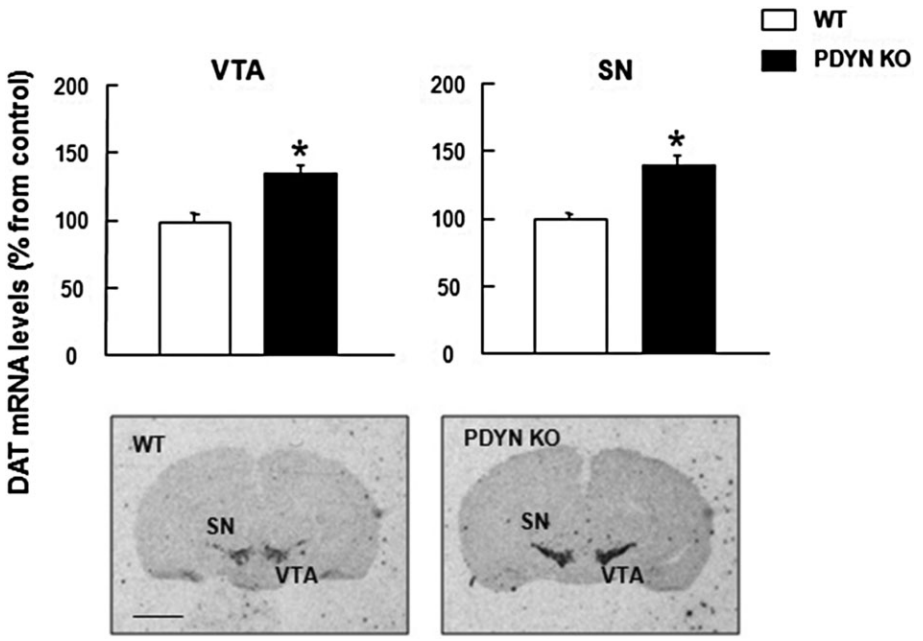

Figure 6 Dopamine transporter (DAT) gene expression in the ventral tegmental area (VTA) and substantia nigra (SN) in prodynorphin knockout (PDYN KO) and wlld-type (WT) mice ($n = 10$). Columns represent the percentage of mean arbitrary units of optical density. *, Values of DAT mRNA levels that differ significantly ($P < 0.05$, Student's *t*-test) from WT mice. The lower panels show the representative autoradiograms of coronal brain sections at the level of the VTA and SN. (Bar represents 1 mm.)

functional activity of the δ-opioid receptor revealed that DPDPE-stimulated [S^{35}]-GTPγS binding was significantly higher in PDYN KO compared with WT mice in the CPu (49%; $t = 4.557$; $P < 0.001$), AcbC (36%; $t = 0.98$; $P = 0.347$), AcbSh (21%; $t = 0.787$; $P = 0.454$) and Cg (44%; $t = 2.23$; $P = 0.047$) (Fig. 9). Moreover, the functional activity of κ-opioid receptor was also significantly higher in PDYN KO compared with WT mice in the CPu

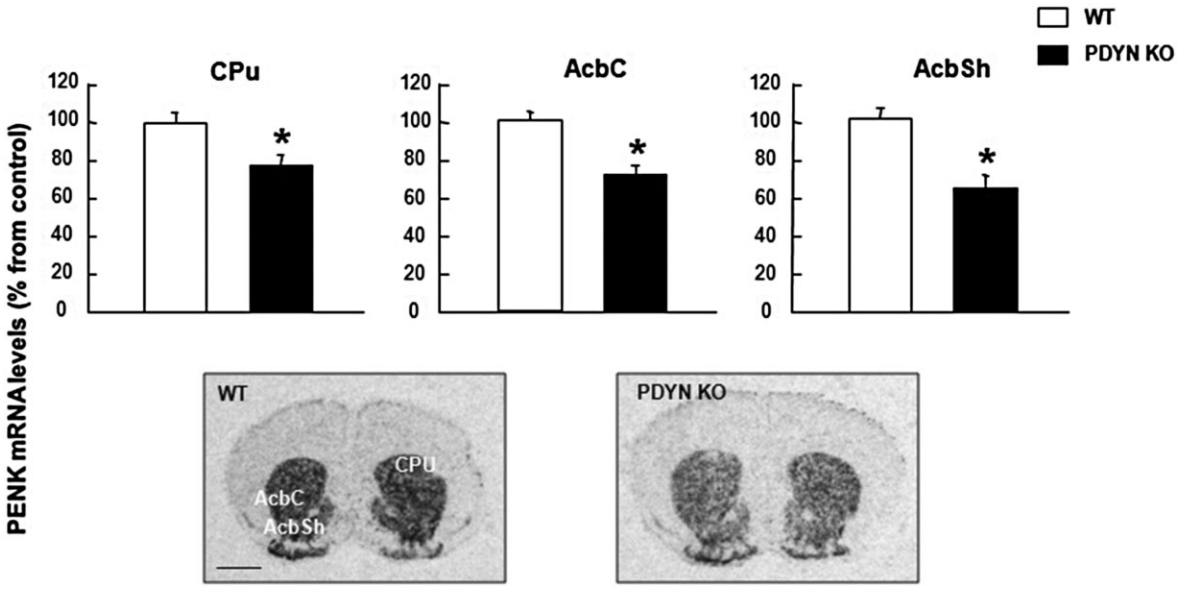

Figure 7 Proenkephalin (PENK) gene expression in the caudate–putamen (CPu) and nucleus accumbens core (AcbC) and accumbens shell (AcbSh) in prodynorphin knockout (PDYN KO) and wlld-type (WT) mice ($n = 10$). Columns represent the percentage of mean arbitrary units of optical density. *, Values of PENK mRNA levels that differed significantly ($P < 0.05$, Student's *t*-test) from WT mice. The lower panels show the representative autoradiograms of coronal brain sections at the level of CPu, AcbC and AcbSh. (Bar represents 1 mm.)

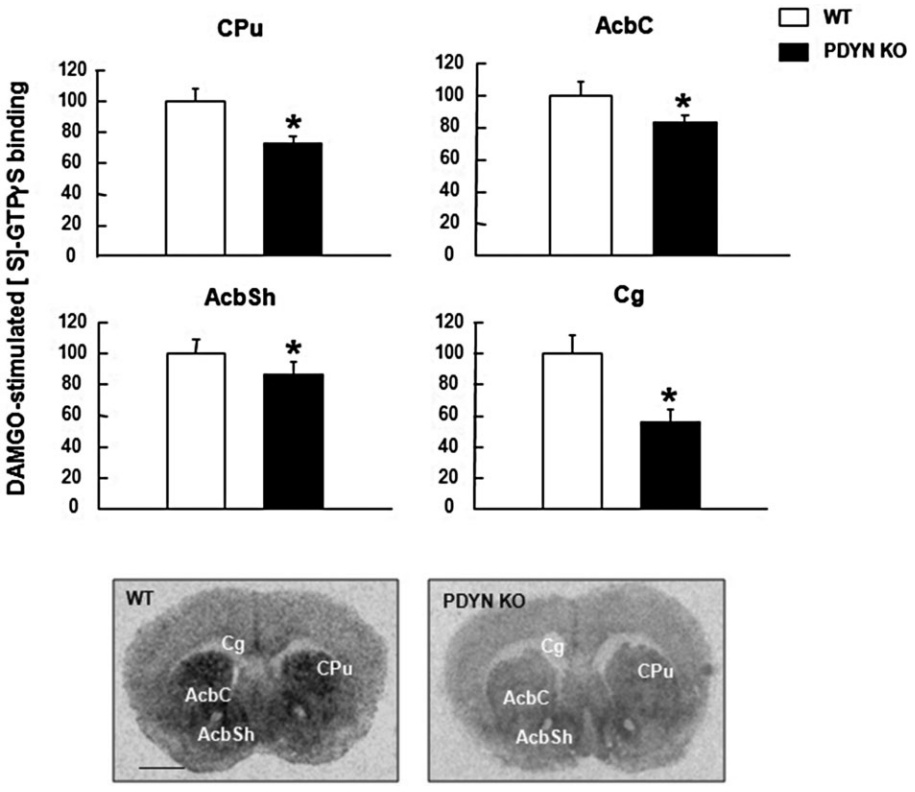

Figure 8 Functionality of the μ-opioid receptor using D-Ala2, N-MePhe4, Gly-ol-enkephalin (DAMGO)-stimulated [S^{35}]-guanosine 5′-3′thiotriphosphate (GTPγS) binding in the caudate–putamen (CPu), nucleus accumbens core (AcbC), nucleus accumbens shell (AcbSh) and cingulate cortex (Cg) of prodynorphin knockout (PDYN KO) and wlld-type (WT) mice ($n = 10$). Columns represent the percentage of mean arbitrary units of optical density. *, Values of DAMGO–stimulated [S^{35}] GTPγS binding in PDYN KO mice that differ significantly ($P < 0.05$, Student's *t*-test) from WT mice. The lower panels show representative autoradiograms of coronal brain sections at the level of the CPu, AcbC, AcbSh and Cg. (Bar represents 1 mm.)

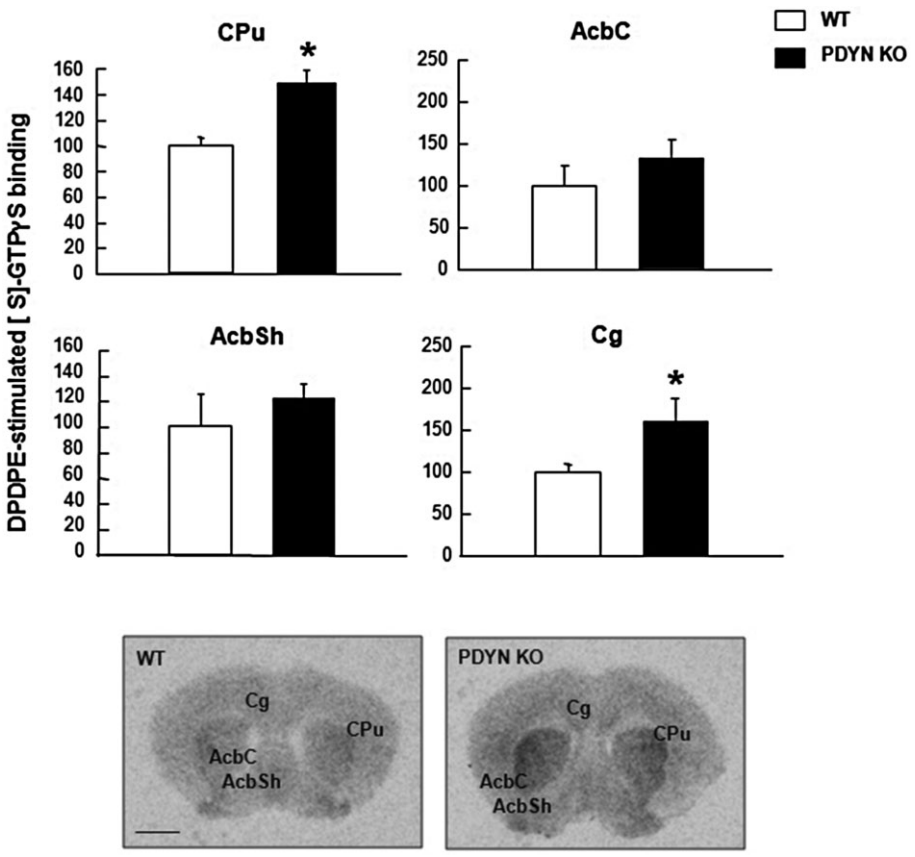

Figure 9 Functionality of the δ-opioid receptor using [D-Pen,D-Pen5]enkephalin (DPDPE)-stimulated [S^{35}]-guanosine 5′-3′thio-triphosphate (GTPγS) binding in the caudate putamen (CPu), nucleus accumbens core (AcbC), nucleus accumbens shell (AcbSh) and cingulate cortex (Cg) of prodynorphin knockout (PDYN KO) and wlld-type (WT) mice ($n = 10$). Columns represent the percentage mean arbitrary units of optical density. *,Values of DPDPE–stimulated [S^{35}] GTPγS binding in PDYN KO mice that differ significantly ($P < 0.05$, Student's *t*-test) from WT mice. The lower panels show representative autoradiograms of coronal brain sections at the level of the CPu, AcbC, AcbSh and Cg. (Bar represents 1 mm.)

(58%; $t = 2.24$; $P = 0.049$), AcbC (39%; $t = 3.006$; $P = 0.010$), AcbSh (41%; $t = 2.48$; $P = 0.029$) and Cg (34%; $t = 2.58$; $P = 0.019$) (Fig. 10).

DISCUSSION

The results of the present study provide evidence that the PDYN gene contributes to vulnerability for ethanol consumption. Several observations were made: (1) PDYN KO mice compared with WT mice exhibited higher CPP for ethanol and higher voluntary ethanol consumption and preference for ethanol; (2) these behavioral alterations were associated with increased TH and decreased DAT mRNA levels in the VTA and SN and decreased PENK gene expression in the CPu, AcbC and AcbS, as well as decreased functional activity of the μ-opioid receptor and increased functional activity of the δ- and κ-opioid receptors in the CPu, AcbC and AcbS Cg.

Several studies have suggested a negative correlation between hypnotic LORR with ethanol and voluntary ethanol consumption (Thiele *et al.* 1998; Hodge *et al.* 1999), and an inverse correlation between withdrawal severity and alcohol consumption (Metten *et al.* 1998). However, in agreement with Blednov *et al.* (2006), the present study showed that PDYN gene deletion did not change either the hypnotic effects of ethanol or withdrawal severity. Further, hypothermic effects after the acute administration of ethanol have been well documented in rodents (Erwin, Jones & Radcliffe 1990). This measure is useful for studying the physiological response to ethanol in mice (Radcliffe *et al.* 2005). In this study, PDYN mice and WT mice did not differ from each other in their hypothermic responses to an acute ethanol challenge (3 g/kg, p.o.), probably because ethanol-induced hypothermia is a polygenic trait, as has been postulated (Erwin *et al.* 1990). Studies support the idea that oral ethanol intake is modulated by ethanol-induced hypothermia (Cunningham & Niehus 1989) and could be inversely related with the magnitude of ethanol-induced hypothermia and genetically influenced (Cunningham *et al.* 1991). However, other authors have demonstrated that selected drinking and non-drinking rats differ in

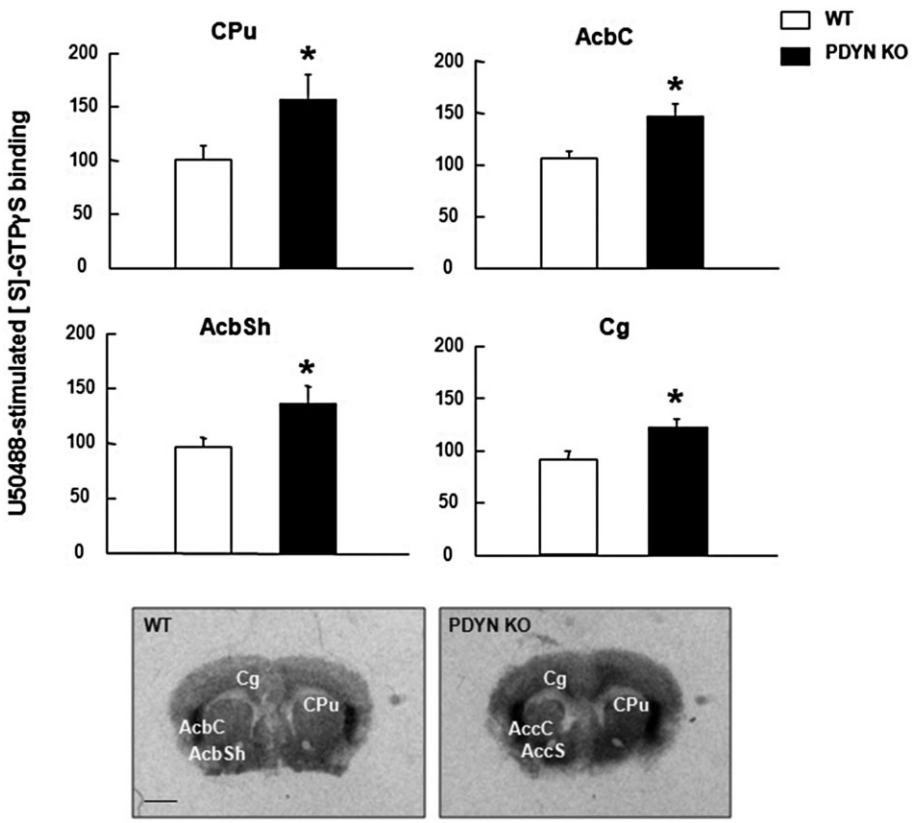

Figure 10 Functionality of the κ-opioid receptor using U50448-stimulated [S^{35}]-guanosine 5′-3′thio-triphosphate (GTPγS) binding in the caudate–putamen (CPu), nucleus accumbens core (AcbC), nucleus accumbens shell (AcbSh) and cingulate cortex (Cg) of prodynorphin knockout (PDYN KO) and wlld-type (WT) mice ($n = 10$). Columns represent the percentage mean arbitrary units of optical density. *, Values of U50,448H–stimulated [S^{35}] GTPγS binding in PDYN KO mice that differ significantly ($P < 0.05$, Student's *t*-test) from WT mice. The lower panels show the representative autoradiograms of coronal brain sections at the level of the CPu, AcbC, AcbSh and Cg. (Bar represents 1 mm.)

their ethanol intake behavior but not in their initial sensitivity to ethanol (Daoust *et al.* 1987). Moreover, in Wistar rats predisposed to develop high ethanol intake in a free-choice condition, hypothermia did not predict increased vulnerability to acquire high ethanol preference (Bisaga & Kostowski 1993). Therefore, the sensitivity of ethanol-induced hypothermia may not necessarily be related to increased ethanol intake/self-administration.

Blood ethanol concentrations were higher (10%) in PDYN KO than in WT mice after acute ethanol administration (3 g/kg p.o.), suggesting a difference in physiological response that might be related with increased vulnerability to ethanol consumption. In fact, several studies have suggested that elevation of BELs may be related with increased ethanol consumption. For example, C57BL6 mice exposed to ethanol present a significant elevation in BELs (Becker & Lopez 2004). Moreover, Le *et al.* (1994) measured ethanol consumption in several strains of mice in a limited-access paradigm and found that the C57BL6 strain mice had more ethanol consumption and that this finding was associated with higher blood ethanol concentrations.

Low dynorphin concentration in the NAcc may motivate drinking because this peptide, acting via κ-opioid receptors, inhibits DA release (Di Chiara & Imperato 1988b). In fact, lower levels of dynorphin peptides were found in the NAcc of ethanol-preferring alcohol-preferring (AA) rats compared with ethanol-avoiding alcohol-avoiding (ANA) rats (Nylander *et al.* 1994). However Gulya *et al.* (1993) demonstrated that chronic ethanol ingestion in C57BL6 mice increased PDYN gene expression in several brain regions, including the hypothalamus, hippocampus, NAcc, striatum, septum and olfactory tubercle. Furthermore, higher PDYN levels were found in the AcbSh of rats with high avoidance acquisition in the two-way shuttle box (RHA) showing higher ethanol intake and preference (Guitart-Masip *et al.* 2006), whereas no differences were found in PDYN gene expression in several brain regions, including the NAcc, CPu and amygdala in fawn-hooded rats after chronic free-choice ethanol consumption (Cowen & Lawrence 2001). These variations in PDYN gene expression may be because of the different species (mice or rat) and ethanol-drinking paradigms studied. On the other hand, the removal of the DA release inhibitory tone mediated by

κ-opioid receptors and dynorphins (Koob 1992a) might facilitate the reinforcing properties of ethanol and increase consumption. Thus, the selective κ-opioid receptor agonist U50,488H reduces voluntary ethanol intake in rats (Lindholm *et al.* 2001), whereas the blockade of κ-opioid receptors by the antagonist nor-binaltorphimine increases ethanol consumption in rats (Mitchell *et al.* 2005). However, mice lacking κ-opioid receptors reduce ethanol consumption (Kovacs *et al.* 2005). The present study showed that deletion of the PDYN gene significantly increased ethanol-conditioned place preference, as well as ethanol consumption and preference in a two-bottle free-choice paradigm. In contrast, Blednov *et al.* (2006) find no differences in the rewarding properties of ethanol, as measured by the CPP of male PDYN KO mice versus their WT littermates for either ethanol consumption or ethanol preference according to the two-bottle choice protocol. On the other hand, alcoholism has recently been associated with PDYN-GpG-methylation, activation of PDYN transcription and increased vulnerability to alcohol dependence (Taqi *et al.* 2011). The effect of mutations on voluntary ethanol consumption seems to be strongly dependent on the genetic background of mice. The genes in the background strains on which the mutation is placed are important for results (Crabbe *et al.* 2006). Therefore, the discrepancies observed in ethanol consumption and preference may depend, at least in part, on the genetic background: C57Bl/6 (present study) versus mixed genetic background C57Bl/6 × 129/SvEv-Tac (Blednov *et al.* 2006), and/or differences in the specific experimental method used (ethanol dose or concentration, method of detection, or the precision of the method used to measure the volume of ethanol intake).

The distinct vulnerability for ethanol consumption found in PDYN KO mice may be related to functional alterations in key neurochemical targets that regulate reinforcement and reward. DA plays a major role in mediating the motivational effects of substances of abuse (including ethanol) in the mesolimbic system (Wise & Bozarth 1982; Wise 1987, 1996; Di Chiara & Imperato 1988a; Koob 1992b). Ethanol enhances the firing rate of DA neurons in the VTA (Gessa *et al.* 1985; Brodie, Shefner & Dunwiddie 1990; Brodie & Appel 1998) and significantly increases the release of DA in different brain regions (Di Chiara & Imperato 1985, 1988a; Imperato and Di Chiara, 1986; Weiss *et al.* 1993) after distinct patterns of ethanol administration. The DAT provides a large measure of regulation of synaptic DA levels (Vandenbergh *et al.* 2000). Within this reward system, DAT plays an important role in the regulation of dopaminergic neurotransmission via pre-synaptic reuptake (Lind, Eriksson & Wilhelmsen 2009). The results of the present study indicate that DAT gene expression was higher and TH gene expression was lower in the VTA and SN of PDYN KO mice compared with WT mice. Chefer *et al.* (2005) found increased DA levels in mice lacking κ-opioid receptor or treated with a κ-opioid receptor antagonist, suggesting that this endogenous opioid system produces dynamic regulation of mesolimbic DA (Chefer *et al.* 2005). Thus, changes in DAT and TH gene expression stabilize DA availability in the synaptic cleft of neurons.

A large body of evidence suggests that ethanol consumption is regulated in part by the opioid system (Ulm, Volpicelli & Volpicelli 1995; Froehlich 1996; Herz 1997). Ethanol consumption induces the release of opioid peptides to stimulate key brain regions closely involved with reward and positive reinforcement (Ulm *et al.* 1995; Froehlich 1996; Jamensky & Gianoulakis 1999). Moreover, ethanol consumption produces changes in the expression of opioid peptides and receptors, leading to 'neuroadaptations' that may increase vulnerability to ethanol consumption and the development of dependence (Manzanares *et al.* 2005; Ron & Jurd 2005). In fact, baseline differences in opioid gene expression have been related with a greater predisposition to increased ethanol intake (Nylander *et al.* 1994; Gianoulakis, de Waele & Thavundayil 1996; Fadda *et al.* 1999; Jamensky & Gianoulakis 1999; Marinelli, Kiianmaa & Gianoulakis 2000; Manzanares *et al.* 2005; Femenía *et al.* 2009). In the present study, PDYN gene deletion significantly decreased PENK gene expression in the CPu, nucleus AcbC and AcbSh, and Cg. It can be hypothesized that these changes occur because dynorphin opioid peptides tonically inhibit PENK gene expression. Therefore, removal of this inhibitory action would result in elevation of PENK gene expression, contributing, at least in part, to increased vulnerability to ethanol consumption.

The μ-opioid receptor has been largely related with increased vulnerability to ethanol consumption, the development of ethanol dependence and relapse, and, therefore, one of the most important therapeutic targets for controlling ethanol dependence and craving (Manzanares *et al.* 2005). Differences in μ-opioid receptor densities were reported in rats presenting high and low preference for ethanol consumption (Cowen *et al.* 1999; Fadda *et al.* 1999; Sim-Selley *et al.* 2002). Acute administration of ethanol increases PENK gene expression in the CPu, AcbC, AcbSH and Cg (Oliva *et al.* 2008), whereas chronic ethanol consumption down-regulates μ-opioid receptor function in Wistar rats (Oliva & Manzanares 2007), ethanol-preferring rats (Chen & Lawrence 2000) and in the NAcc and CPu of Wistar rats (Turchan *et al.* 1999). In contrast, Sardinian rats present increased μ-opioid receptor binding in the CPu (Fadda *et al.* 1999). In this study, the results revealed significant down-regulation of the μ-opioid receptor in PDYN KO mice. In contrast, Clarke *et al.* (2003) find up-regulation of the μ-opioid receptor in PDYN KO mice (Clarke *et al.* 2003).

These discrepancies may be due to differences in the experimental procedure, the genetic construction used to delete the PDYN gene or the specific strain of mice. On the other hand, the δ- and κ-opioid receptors were significantly up-regulated in PDYN KO mice (CPu, Acb and Cg). The enkephalins bind with greater affinity to δ-opioid receptors and the dynorphins tend to bind selectively to κ-opioid receptors. This compensatory up-regulation of the δ- and κ-opioid receptors probably occurs as a consequence of lower levels of PENK gene expression and the lack of dynorphins. Accordingly Clarke *et al.* (2003) also found a compensatory up-regulation of δ- and κ-opioid receptors in the same regions of PDYN KO mice. In addition, Chefer & Shippenberg (2006) suggest that up-regulation of the κ-opioid receptor is functional because the inhibitory effects of the selective κ-opioid receptor agonist U69593 on the NAcc dialysate of DA are enhanced in PDYN KO mice. These alterations found in the opioid receptors of PDYN KO mice suggest a tonic regulatory role of the dynorphin peptides in the modulation of opioid receptor expression.

Taken together, the current study suggests that the PDYN gene plays an important role in the regulation of alcohol consumption. The study showed that lack of the PDYN gene is associated with an increase in ethanol preference and consumption, changes in PENK, TH, and DAT expression, and changes in the functional activity of the μ-, δ-, and κ-opioid receptors. These data suggest that the PDYN gene may contribute to the development of alcohol use disorder through its influence on the dopaminergic and opioid systems.

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Authors Contribution

TF and JM were responsible for the study concept and design. TF contributed to the acquisition of animal data, performed the experiments and data analysis, and assisted with the interpretation of findings. TF and JM drafted the manuscript and provided critical revision of the manuscript for important intellectual content. Both authors critically reviewed content and approved the final version for publication.

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