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NEUROPEPTIDE Y-Y2 RECEPTOR KNOCKOUT MICE: INFLUENCE OF GENETIC BACKGROUND ON ANXIETY-RELATED BEHAVIORS

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Abstract—Neuropeptide Y (NPY) has been extensively studied in relation to anxiety and depression but of the seven NPY receptors known to date, it is not yet clear which one is mainly involved in mediating its effects in emotional behavior. Mice lacking the NPY-Y2 receptors were previously shown to be less anxious due to their improved ability to cope with stressful situations. In the present study, the behavioral phenotype including the response to challenges was analyzed in NPY-Y2 knockout (KO) mice backcrossed in to congenic C57BL/6 background. In the elevated plus-maze and the forced swim test (FST), the anxiolytic-like or antidepressant-like phenotype of the NPY-Y2 knockout mice could not be confirmed, although this study differs from the previous one only with regard to the genetic background of the mice. In addition, no differences in response to acute stress or to the antidepressant desipramine in the forced swim test were detected between wild type and NPY-Y2 knockout animals. These results suggest that the genetic background of the animals appears to have a strong influence on the behavioral phenotype of NPY-Y2 knockout mice. Additionally, to further characterize the animals by their biochemical response to a challenge, the neurochemical changes induced by the anxiogenic compound yohimbine were measured in the medial prefrontal cortex (mPFC) of NPY-Y2 knockout and compared to wild type mice. Dopamine (DA) levels were significantly increased by yohimbine in the wild type but unaffected in the knockout mice, suggesting that NPY-Y2 receptor exerts a direct control over both the tonic and phasic release of dopamine and that, although the anxiety-like behavior of these NPY-Y2 knockout mice is unaltered, there are clear modifications of dopamine dynamics. However, yohimbine led to a significant increase in noradrenaline (NA) concentration and a slight reduction in serotonin concentration that were identical for both phenotypes.

Key words: depression, stress, yohimbine, dopamine, noradrenaline, serotonin.

Preclinical and clinical studies suggest that neuropeptide Y (NPY) has a major role in the pathophysiology of affective disorders such as depression and anxiety; in particular, a general down-regulation of this neurotransmitter has been demonstrated in various animal models of depression and anxiety and in human depressed patients (Heilig, 2004; Heilig et al., 2004).

Of the seven receptor subtypes discovered to date, NPY-Y1/Y7, the main receptor subtypes mediating emotionality-related behaviors in rodents are NPY-Y1, -Y2 and -Y5 that are differentially expressed in specific brain regions (Larsen et al., 1993; Dumont et al., 1996, 1998; Gerald et al., 1996; Caberlotto et al., 1997, 1998; Gustafson et al., 1997). In particular NPY-Y2 receptors, which are primarily presynaptic (King et al., 1999), have been demonstrated to have a role in the regulation of NPY functions in emotional behavior and stress response, although the anxiolytic-like effects of NPY are likely more related to downstream effects at the postsynaptic NPY-Y1 receptors (Heilig, 1995; Karlsson et al., 2008). The role of NPY-Y2 receptors has been supported by evidence of an anxiogenic-like effect observed in the elevated plus maze (EPM) following i.c.v. injection of the NPY-Y2/Y5 preferring agonist $NPY_{13–36}$ (Nakajima et al., 1998). Moreover, intra-amygdaloid injections of C2-NPY, a selective NPY-Y2 receptor agonist, induce increased anxiety (Sajdyk et al., 2002), although injections of $NPY_{13–36}$ close to the locus coeruleus can induce an anxiolytic-like response in the social interaction test and in the EPM (Kask et al., 1998). AQ: 2

Oppositely, an anxiolytic-like profile has been obtained through administration of the NPY-Y2 selective antagonist BIIE0246, which increased the time rats spent in the open arms of the EPM (Bacchi et al., 2006), or through a selective deletion of NPY-Y2 receptors in the central and basolateral amygdala, which is probably due to a downstream effect on NPY-Y1 receptors (Tasan et al., 2010). NPY-Y2 receptors have also been linked to the action of antidepressant drugs, since chronic imipramine treatment reduced NPY binding to the NPY-Y2 receptor subtype in discrete rat brain regions (Widdowson and Halaris, 1991).

Furthermore, previous studies have investigated the depressive- and anxiety-related behaviors in mice lacking NPY-Y2 receptors, which have displayed reduced immobility in the forced swim test (FST), supporting a role for NPY-Y2 receptor in depressive behavior and increased time spent in the open arms of the EPM compared to their wild type (WT) littermates (Tschenett et al., 2003; Carvajal

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Abbreviations: ACTH, adrenocorticotropin hormone; ANCOVA, analysis of covariance; ANOVA, analysis of variance; AUC, area under the curve; DA, dopamine; EPM, elevated plus maze; FST, forced swim test; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; KO, knockout; LABORAS, laboratory animal behavior observation registration and analysis system; mPFC, medial prefrontal cortex; NA, noradrenaline; NPY, neuropeptide Y; n.s., not significant; PCR, polymerase chain reaction; WT, wild type.

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et al., 2006), indicating a role for NPY-Y2 receptor in the modulation of anxiety. Overall, these data suggest that NPY-Y2 blockade in the NPY-Y2 knockout (KO) mice is related to the animals' ability to better cope with stressful situations.

In the present study, to further investigate the role of NPY-Y2 receptors in emotional behavior and stress response, the mixed C57BL/6-129SvJ genetic background NPY-Y2 KO mice backcrossed with pure C57BL/6 strain mice were characterized together with their WT littermates in a series of behavioral tests of anxiety and depression in basal conditions and following exposure to acute stress or antidepressant treatment with desipramine. Additionally, the neurochemical substrate sustaining the presumed anxiolytic-like effects of NPY-Y2 blockade in KO mice was considered in these mice. Therefore dopamine (DA), noradrenaline (NA) and serotonin (5-HT) effluxes were measured in freely moving animals in the medial prefrontal cortex (mPFC) following administration of the alpha-2 adrenoceptor antagonist yohimbine—a potent anxiogenic compound (Charney et al., 1989)—in both WT and NPY-Y2 KO mice.

EXPERIMENTAL PROCEDURES

Animals

Mixed C57BL/6-129SvJ genetic background NPY-Y2 KO mice were generated at the Garvan Institute of Medical Research (Sydney, New South Wales, Australia) using the cre/loxP technology to delete the entire coding sequence of the NPY-Y2 receptor (Sainsbury et al., 2002). The mice were successively backcrossed eight generations onto a C57BL/6 background in our facility, obtaining NPY-Y2 KO mice with congenic C57BL/6 genetic background. As controls, C57BL/6 WT mice in which the deletion of NPY-Y2 gene was not present were used in all the experiments.

In vitro experiments

To demonstrate the presence of NPY-Y2 receptor gene in the WT mice and its absence in the NPY-Y2 KO mice, a series of *in vitro* analysis was performed. Reverse transcriptase-polymerase chain reaction (PCR) using different couples of oligonucleotide primers recognizing the mouse NPY-Y2 receptor gene was performed on a cDNA substrate obtained from hippocampal tissue of both WT and NPY-Y2 KO mice (Fig. 1A), since NPY-Y2 receptor has been demonstrated to be highly expressed in this brain region (Dumont et al., 1996). Receptor autoradiography was performed using 25 pM [^{125}I]PYY radioligand (Amersham Biosciences, Milano, Italy), which unselectively binds all the NPY receptors (Gobbi et al., 1998), on 14 μm-thick coronal brain sections collected approximately at −1.34 mm from Bregma level (Paxinos and Franklin, 1997; Fig. 1B). Finally, on the same collection of brain sections, *in situ* hybridization reaction was carried out as previously described (Zambello et al., 2008) using a riboprobe complementary to rat NPY-Y2 mRNA, which displays 95% sequence homology with mouse NPY-Y2 mRNA (data not shown).

Real-time quantitative PCR. To verify the presence of possible differences between C57BL/6 and 129SvJ strains in the basal NPY mRNA levels in the hypothalamus, a brain region in which the highest NPY levels have been observed (Morris, 1989), a real-time quantitative PCR was performed using hypothalamic samples from C57BL/6 and 129SvJ male mice (n=5). Primers, probes and standards specific for mouse NPY and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) gene, used as control housekeeping gene, were synthesized by Proligo, Europe. The reactions were performed in triplicate in 96-well optical plates (Applied Biosystems) using TaqMan® Universal PCR Master Mix kit (Applied Biosystems), following manufacturer's instructions. The plates were analyzed with ABI PRISM 7900HT Fast sequence detector (Applied Biosystems), as specified in manufacturer's instructions. Data were acquired and processed with SDS software v2.3 (Applied Biosystems).

Behavioral experiments

Adult (9–12 weeks old) male and female NPY-Y2 KO mice and their WT controls were used in the present study. They were kept under standard 12 h light (6:00 AM–6:00 PM) and 12 h dark (6:00 PM–6:00 AM) cycles, with food and water *ad libitum*. In two separate rooms, male and female mice were individually caged, in order to control the variability otherwise induced by the establishment and maintenance of social hierarchy occurring in group-housed male mice. Mice were allowed to habituate to the testing room for at least 1-h before performing the behavioral tests. All the tests were performed between 9:00 AM and 3:00 PM and test environments were accurately cleaned between each test session.

All the procedures involving the care of the animals and the experimental protocols were conducted in accordance with the European Communities Council Directive of 24 November, 1986 (86/609/EEC) and the Ministry of Health DL116/1992, Roma, and in accordance with GlaxoSmithKline ethical standards. All efforts were made to minimize the number of experimental animals used and their suffering.

LABORAS^{TM} system. The laboratory animal behavior observation registration and analysis system (LABORAS^{TM}) system (Metris B.V., Hoofddorp, The Netherlands) was used to automatically record different animal movements and translating them into corresponding behavioral categories, such as feeding, drinking, climbing, grooming, locomotor activity and total distance, as previously described (Van de Weerd et al., 2001).

The NPY-Y2 KO and WT male (n=15–16) and female (n=8) mice were habituated to the LABORAS^{TM} cages 2 h before the beginning of the recording, which was then performed for 24 h.

Elevated plus maze (EPM). The EPM is a validated test of anxiety (Pellow and File, 1986), consisting of a platform positioned 50 cm above ground, constituted of two opposing open arms (30×5 cm) and two opposing closed arms of the same size. The test was conducted in a red dim light (25 lx in the closed arms; approximately 100 lx in the open arms). Each mouse was initially placed on the central part of the maze facing an open arm. Mice were randomly tested and their behavior was video recorded and then analyzed by an observer blind to the genotype. The percentage of time spent onto the open and closed arms and the number of entries into the open arms were determined. The EPM was performed for 5 min in male NPY-Y2 KO and WT mice (n=19); some of the animals (n=9–10) were previously stressed with 5 min of restraint, by placing them in a Plexiglas tube, and then immediately transferred to the maze; some others (n=9–10) were not previously stressed.

Forced swim test (FST). The FST is a validated test to examine the depressive-like state of the animals (Porsolt et al., 1977). In the present study, the FST was performed following two different modalities: the first one was conducted on male NPY-Y2 KO and WT mice (n=8) tested with the LABORAS^{TM} system 10 days before. Each mouse was placed in an open container (diameter 10 cm, height 25 cm) filled with 10 cm deep tap water maintained at 25 °C; its activity was video recorded over a period of 6 min and the total time of immobility was measured during the last 4 min by an observer blind to the genotype.

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Fig. 1. (A) Agarose gel electrophoresis results from reverse-transcriptase PCR. Two 303-base pairs bands (column a and e) and a 489-base pairs band (column i) in the agarose gel correspond to NPY-Y2 receptor sequences obtained from three different couples of PCR primers. The bands were obtained from RT-PCR reactions in which hippocampal cDNAs from WT mice were used as substrates and reverse transcriptase enzyme was added to the reactions. In the columns in which no bands were visible in the agarose gel, hippocampal cDNAs from NPY-Y2 KO mice were used as substrates and/or reverse transcriptase enzyme was not added to the reactions. The experiment showed that NPY-Y2 receptor gene was found only in the WT mice, not in the NPY-Y2 KO. (B) Receptor autoradiography for NPY-Y2 receptors. Representative images of mouse coronal brain sections, approximately at –1.34 mm from Bregma. Effect of total binding obtained incubating with 25 pM of the ligand [^{125}I]PYY the WT (i) and the NPY-Y2 KO (iii) mouse brain sections. Effect of non specific binding, obtained adding 1 μM of non radioactive NPY to [^{125}I]PYY in the WT (ii) and in the NPY-Y2 KO (iv) mice. A specific labelling is present in the WT mice, but not in the NPY-Y2 KO, whereas the lack of NPY-Y2 receptor signal due to non specific labelling is observed both in the WT and in the NPY-Y2 KO mice. Scale bar=2.5 mm.

The second modality was applied both in male and female NPY-Y2 KO and WT mice (n=8) previously exposed to the LABORAS^{TM} system and EPM, which were placed in a container (diameter 18 cm, height 35 cm) filled with 15 cm deep tap water maintained at 25 °C; their activity was videotaped for 10 min and the immobility time was considered during the last 5 min. Mice were considered to be immobile when floating passively in the water, maintaining only those movements necessary to keep them floating.

FST after desipramine treatment. The FST was also performed in male (n=8) and female (n=10–11) NPY-Y2 KO and WT mice, before injected i.p. with two different doses (10 mg/kg and 20 mg/kg) of the antidepressant desipramine hydrochloride (Sigma-Aldrich, Milano, Italy), whereas control animals were i.p. injected with vehicle (saline solution). The injections were performed 30 min before the beginning of the tests, in which a container (18 cm diameter, 35 cm height) filled with 15 cm deep tap water at 25 °C was used. The FST was conducted over a period of 10 min and the immobility time was considered between the second and the sixth minute.

Adrenocorticotropin hormone (ACTH) and corticosterone levels after FST at 21 °C. The FST conducted in a container (diameter 10 cm, height 25 cm) filled with 10 cm deep tap water (21 °C) was applied as stress. It was performed only in male NPY-Y2 KO and WT mice (n=16). The mice were individually placed in the container for 5 min, while NPY-Y2 KO and WT controls (n=16) were maintained into their home cages. Five minutes after the stress procedure, mice were sacrificed by decapitation. Blood samples were collected from the animals and adrenocorticotropin hormone (ACTH) and corticosterone levels were measured using specific radioimmunoassay kits (IRMA; Immunoradiometric Assay, DiaSorin, Saluggia, Italy) and RIA (Radioimmuno Assay, MP Biomedicals, Orangeburg, NY, USA), respectively.

Microdialysis

Adult male C57BL/6 WT and NPY-Y2 KO mice were anesthetized by administration of medetomidine hydrochloride (0.04 ml/100 g, s.c., Domitor®, Pfizer, USA), followed by a mixture of tiletamine and Zolazepam (50 mg/ml/kg, Zoletil®, Virbac, France). At the

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end of surgery, anaesthesia was reversed by administration of 0.1 mg/kg s.c. administration of atipamezole (Antisedan, Pfizer, USA). Analgesia was provided by s.c. administration of the non-steroidal anti-inflammatory Rymadil® (5 mg/kg, Pfizer, USA). Once anesthetized, mice were placed in a stereotaxic apparatus (David Kopf), the skull was exposed and a MAB 4.9 guide cannula was inserted above the mPFC at the following coordinates (in mm from Bregma): anterior+2.2, lateral+0.3, ventral −1.0 according to Franklin and Paxinos (2003). After 2 days of recovery from surgery, a microdialysis probe (2 mm active membrane, Agnthos, Stokholm, Sweden) was inserted through the guide cannula. The following day, probe was perfused with artificial cerebrospinal fluid (composition in mM: KCl 2.5, NaCl 125, $CaCl_2$ 1.3, $MgCl_2$ 1.18, Na_2HPO_4 adjusted at pH 7.4 with H_3PO_4 85%) at a steady flow rate (1.1 μl/min) with a microinfusion pump (U-864, Univentor, Agnthos, Stokholm, Sweden). Samples were collected every 20 min in 3 μl oxalic acid (1 mM) throughout the experiment. A 2-h period of equilibration was allowed before collection of three basal samples. Mice received a first injection of vehicle (saline) followed, an hour later, by yohimbine (2.5 mg/kg s.c.) and sampling went on for other 3 h. All samples were frozen and stored at −80 °C for subsequent analysis for monoamines concentration.

AQ: 3 Microdialysates were analysed with HPLC coupled with electrochemical detection (Decade detector, ANTEC, Leyden, NL) with a VT-03 flow cell (Voltage: 380 mV). A mobile phase composed of $NaCH_3COO$ (1.0 M), NaCl (0.5 M), EDTA (0.1 mM) and methanol (20%) was pumped at a flow rate of 0.32 ml/min. Separation was performed using a Phenomenex Capcell Pak 55 SCX UG80 150×1.5 mm column (Heidbreder et al., 2001). NA, DA and 5-HT concentrations in samples were measured against that of external standard solutions.

At the end of experiment, animals were sacrificed with CO_2. A punctured microdialysis probe was inserted in the cannula and flushed with a blue dye solution (0.1 μl). Brains were then quickly removed and the microdialysis membrane track identified using a stereomicroscope on coronal sections.

Statistical analysis

The statistical analysis was performed using "Statistica 6.0" software package. The data obtained from real-time quantitative PCR were analyzed as described by Bond et al. (2002) with the analysis of covariance (ANCOVA), considering the expression of GAPDH as a covariate, under the hypothesis that it was not affected by changes in the two different mouse strains; it was then followed by Student's *t*-test between the expression data in the two mouse strains. The data obtained from LABORAS™, EPM and FST studies were analyzed by Student's *t*-test, comparing the two groups of mice, WT and NPY-Y2 KO. In the FST after desipramine treatment, two-way analysis of variance (ANOVA), followed by Tukey–Kramer post hoc test when appropriate was carried out to evaluate the effects of the interaction between treatment and genotype on the immobility time. Moreover, two-way ANOVA was carried out to evaluate the effect of stress (FST at 21 °C) on ACTH and corticosterone levels in the NPY-Y2 KO and WT mice. All the results were expressed as mean±SEM and in all the statistical analysis the *P*-value has been considered significant if lower than 0.05.

For microdialysis studies, the data were not corrected for probe recovery. Crude basal and saline levels were analysed by two-way ANOVA with a between-subjects factor of genotype (WT vs. KO) and repeated measurement factor of time (three 20 min samples). Basal DA levels for the WT group did not stabilize until the injection of saline (see below); hence all results (DA, NA and 5-HT in pg/sample) were transformed into percentage of the average of saline levels. Post-yohimbine results (percent transformed) were analyzed using two-way ANOVA with a between-subjects factor of genotype (WT vs. KO) and repeated measurements factor of time (nine 20 min samples). Post hoc unpaired *t*-tests were run when significance on main factors (genotype, time) or interactions of main factors was reached. Percent values were transformed into area under the curve (AUC) calculated between 40 and 220 min following the Simpson's rule (Abramowitz and Stegun, 1972) and analysed with one-way ANOVA.

RESULTS

In vitro experiments

Real-time quantitative PCR. The statistical analysis of the gene expression data showed that the covariance efficiency factor was close to 1 for GAPDH and NPY (1.085 and 1.195, respectively), thus proving independence of the basal GAPDH mRNA and NPY mRNA expression from the different mouse strains. Student's *t*-test was then performed by comparing the means of the GAPDH mRNA levels expressed on a $\log_{10}$ scale in the two mouse strains (C57BL/6 strain: 6.39±0.06; 129SvJ strain: 6.35±0.01; t_8=0.58, P=0.57, not significant (n.s.)) and the means of NPY mRNA expression (C57BL/6 strain: 5.29±0.17; 129SvJ strain: 5.22±0.05; t_8=0.44, P=0.67, n.s.), which were not different between the two strains. Therefore, no differences of endogenous NPY mRNA levels were found in the hypothalamus between the two mouse strains C57BL/6 and 129SvJ (data not shown).

Behavioral experiments

LABORAS™ system. The basal behaviors of NPY-Y2 KO and WT male and female mice examined with the LABORAS™ system were separately evaluated during the dark phase (6:00 PM–6:00 AM) and the light phase (6:00 AM–6:00 PM) of the day. A significant increase in terms of climbing activity expressed in seconds and in terms of total distance expressed in meters was found in the NPY-Y2 KO male mice compared to the WT during the dark phase (for climbing: t_{29}=3.28, P=0.003; Fig. 2A; for total distance: t_{29}=3.03, P=0.005; Fig. 2B, Table 1). However, no significant differences between WT and NPY-Y2 KO male mice were observed in terms of locomotor activity, grooming, feeding and drinking (for locomotor activity: t_{29}=1.67, P=0.1, n.s.; for grooming: t_{29}=0.86, P=0.4, n.s.; for feeding: t_{29}=1.76, P=0.09, n.s.; for drinking: t_{29}=1.92, P=0.064, n.s.; Table 1). Moreover, a general reduction of all the activities was observed during the light phase both in the NPY-Y2 KO and in the WT male mice, with no significant differences between the two genotypes (for climbing: t_{30}=1.02, P=0.32, n.s.; for total distance: t_{30}=1.09, P=0.28, n.s.; for locomotor activity: t_{30}=0.94, P=0.35, n.s.; for grooming: t_{30}=0.49, P=0.63, n.s.; for feeding: t_{30}=0.56, P=0.58, n.s.; for drinking: t_{30}=1.18, P=0.24, n.s.; Table 1). F2 T1

No significant differences were found between NPY-Y2 KO and WT female mice on the same behavioral categories during both the dark and light phases (dark phase: for climbing: t_{14}=0.61, P=0.55, n.s.; for total distance: t_{14}=0.56, P=0.58, n.s.; for locomotor activity: t_{14}=0.45, P=0.66, n.s.; for grooming: t_{14}=1.64, P=0.12, n.s.; for feeding: t_{14}=1.17, P=0.26, n.s.; for drinking: t_{14}=0.94, P=0.36, n.s.; Table 1). However, all the activities were

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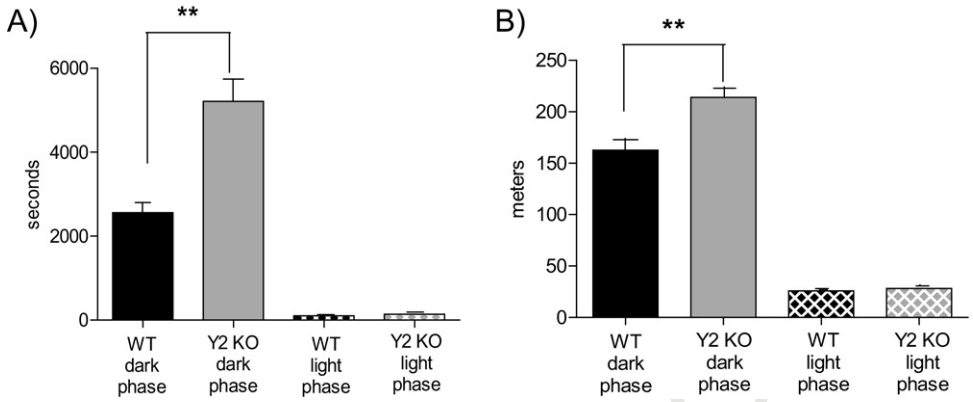

Fig. 2. Graphical expression of two behavioral parameters analyzed with the LABORAS™ system in the WT and NPY-Y2 KO male mice during the dark and light experimental phases: climbing activity (A) and total distance (B). (A) Time, expressed in seconds as mean±SEM, spent in climbing activity: comparison between WT and NPY-Y2 KO male mice (n=15–16) in the dark and light phase of the day; ** P<0.01. (B) Total distance, expressed in meters as mean±SEM: comparison between WT and NPY-Y2 KO male mice (n=15–16) in the dark and light phase of the day; ** P<0.01.

significantly reduced during the light phase compared to the dark phase (light phase: for climbing: t_{14}=1, P=0.33, n.s.; for total distance: t_{14}=0.5, P=0.62, n.s.; for locomotor activity: t_{14}=0.4, P=0.69, n.s.; for grooming: t_{14}=1.93, P=0.07, n.s.; for feeding: t_{14}=0.15, P=0.87, n.s.; for drinking: t_{14}=0.57, P=0.57, n.s.; Table 1).

Elevated plus maze (EPM). In the EPM, no significant differences were found on the percentage of time spent on the open and closed arms of the maze and on the number of entries into the open arms between male WT and NPY-Y2 KO mice (for time in open arms: t_{17}=1.29, P=0.21, n.s.; for time in closed arms: t_{17}=0.68, P=0.5, n.s.; for entries into open arms: t_{17}=0.3, P=0.76, n.s.; Table 2). Additionally, no differences were observed in the EPM between the two groups of mice previously exposed to 5 min of restraint stress (for time in open arms: t_{15}=0.29, P=0.78, n.s.; for time in closed arms: t_{15}=0.39, P=0.7, n.s.; for entries into open arms: t_{15}=0.45, P=0.65, n.s.; Table 2).

Forced swim test (FST). In the FST, no significant differences in terms of immobility time expressed in seconds were found between male and female NPY-Y2 KO and WT mice, both in the 6-min and in the 10-min experiments (FST-6-min in male mice: t_{13}=1.01, P=0.33, n.s.; FST-10-min in male mice: t_{14}=0.57, P=0.58, n.s.; FST-10-min in female mice: t_{14}=0.39, P=0.7, n.s.; Table 2).

FST after desipramine treatment. In male mice, a significant effect of the treatment with desipramine was observed ($F_{2,42}$=4.46, P=0.017). In particular, a reduction of the immobility time expressed in seconds was observed in the WT mice treated with 20 mg/kg desipramine compared to WT mice treated with vehicle (WT-vehicle: 187.4±12; WT-10 mg/kg desipramine: 145.6±19; WT-20 mg/kg desipramine: 94.9±20.2; P=0.034), whereas the treatment with 10 mg/kg desipramine was not significant compared to vehicle (P=0.71; Fig. 3A). Moreover, no significant effects on the immobility time expressed in seconds of 10 mg/kg and 20 mg/kg desipramine treatment

Table 1. Summary of the data collected over 24-h period with the LABORAS™ system in male (n=15–16) and female (n=8) WT and NPY-Y2 KO mice (data are expressed as mean±SEM)

	Dark phase		Light phase	
	WT	Y2 KO	WT	Y2 KO
LABORAS™ system–male mice				
Feeding (s)	2779.22±530.11	4094.20±506.86	787.68±223.99	884.22±131.42
Drinking (s)	144.3±41.02	276.3±55.81	27.01±8.63	38.41±7.72
Climbing (s)	2559.79±381.9	5214.99±729.52**	104.02±34.37	141.45±23.39
Grooming (s)	637.24±238.05	2362.56±212.35	1892.26±172.19	1732.12±179.24
Locom. act (s).	2759.53±205.98	3255.82±215.06	373.83±26.75	408.8±28.71
Tot. distance (m)	162.83±12.41	213.96±11.34**	25.76±2.17	28.2±1.62
LABORAS™ system–female mice				
Feeding (s)	2619.67±785.08	3858.79±715.21	1144.87±571.35	1243.48±276.28
Drinking (s)	112.42±43.09	202.81±86.24	53.28±26.05	72.16±19.88
Climbing (s)	6441.78±914.53	7387.89±1245.17	800.87±578.4	219.59±39.17
Grooming (s)	2709.31±248.5	3364.89±221.78	1897.68±221.78	2848.6±439.87
Locom. act (s)	3169.83±281.18	3373.04±349.54	820.86±365.45	667.14±88.98
Tot. distance (m)	195.38±18.22	209.13±16.34	56.13±26.84	42.32±4.71

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Table 2. Summary of the data from the elevated plus maze (EPM) and the forced swim test (FST) in which no behavioral differences were found between WT and NPY-Y2 KO mice (data are expressed as mean±SEM)

	WT	Y2 KO	WT stress	Y2 KO stress
EPM–male mice				
% Time open arms (s)	15.89±3.5	21.91±2.85	19.33±5.52	20.77±6.47
% Time closed arms (s)	56.55±3.76	52.36±4.71	58.81±4.92	55.29±7.81
N. entries open arms	6.78±1.49	6.20±1.21	3.56±0.6	4.13±1.14
	WT male	Y2 KO male	WT female	Y2 KO female
FST–6 min				
Immobility (s)	190.38±7.71	183.5±3.3	Not performed	Not performed
FST–10 min				
Immobility (s)	284.38±6.02	276.63±12.18	286±7.16	289.5±5.2

EPM: percentage of time (s) spent on the open and closed arms, and number of entries into the open arms. Comparison between WT and NPY-Y2 KO male mice (n=19), not stressed (n=9–10) or previously exposed to 5 min of restraint stress (n=9–10). FST–6 min: comparison between WT and NPY-Y2 KO male mice (n=8) in terms of seconds of immobility calculated during the last 4 min of test. FST–10 min: comparison between WT and NPY-Y2 KO male and female mice (n=8) in terms of seconds of immobility calculated during the last 5 min of test.

compared to vehicle were observed in the NPY-Y2 KO male mice (NPY-Y2 KO-vehicle: 140.4±18.3; NPY-Y2 KO-10 mg/kg desipramine: 122.5±29.6; NPY-Y2 KO-20 mg/kg desipramine: 108.6±21.7; P=0.99 and P=0.89 respectively, n.s.; Fig. 3A). No significant differences between genotypes were observed ($F_{1,42}$=1.22, P=0.27, n.s.) or due to the interaction between genotypes and treatment ($F_{2,42}$=1.08, P=0.35, n.s.).

In female mice, a significant effect of the treatment with desipramine was observed ($F_{2,59}$=12.15, P<0.001). In particular, a significant reduction of the immobility time expressed in seconds was observed in the WT mice treated with 10 mg/kg desipramine compared to the WT vehicle group (P=0.024), whereas the treatment with 20 mg/kg desipramine was not significant (P=0.12, n.s.; WT-vehicle: 173.8±10.1; WT-10 mg/kg desipramine: 92.5±23.6; WT-20 mg/kg desipramine: 107.6±20.5; Fig. 3B). A similar effect on the reduction of the immobility time was observed in the NPY-Y2 KO mice, in which the treatment with 10 mg/kg desipramine was significant compared to vehicle (P=0.03), whereas the treatment with 20 mg/kg was not significant compared to vehicle (P=0.62; NPY-Y2 KO-vehicle: 185±10.2; NPY-Y2 KO-10 mg/kg desipramine: 94.5±18.7; NPY-Y2 KO-20 mg/kg desipramine: 134.2±20.3; Fig. 3B). No significant differences between genotypes were observed ($F_{1,59}$=0.81, P=0.37, n.s.) or due to the genotypes×treatment interaction ($F_{2,59}$=0.24, P=0.79, n.s.).

ACTH and corticosterone levels after FST at 21 °C. Five minutes of FST at 21 °C induced a stressful effect, consisting of a highly significant increase of ACTH ($F_{1,28}$=45.1, P<0.001) and corticosterone ($F_{1,28}$=139.8, P<0.001) levels in plasma and serum respectively, both in WT and NPY-Y2 KO male mice (ACTH levels expressed in pg/ml: WT-no stress: 65.37±13.43; WT-stress: 353.15±65; NPY-Y2 KO-no stress: 79.75±14.23; NPY-Y2 KO-stress: 402.86±60.46; corticosterone levels expressed in ng/ml: WT-no stress: 52.75±7.91; WT-stress: 401.53±44.54; NPY-Y2 KO-no stress: 48.61±7.5; NPY-Y2 KO-stress: 357.56±31.5; data not shown). No significant differences between genotypes were detected: WT and

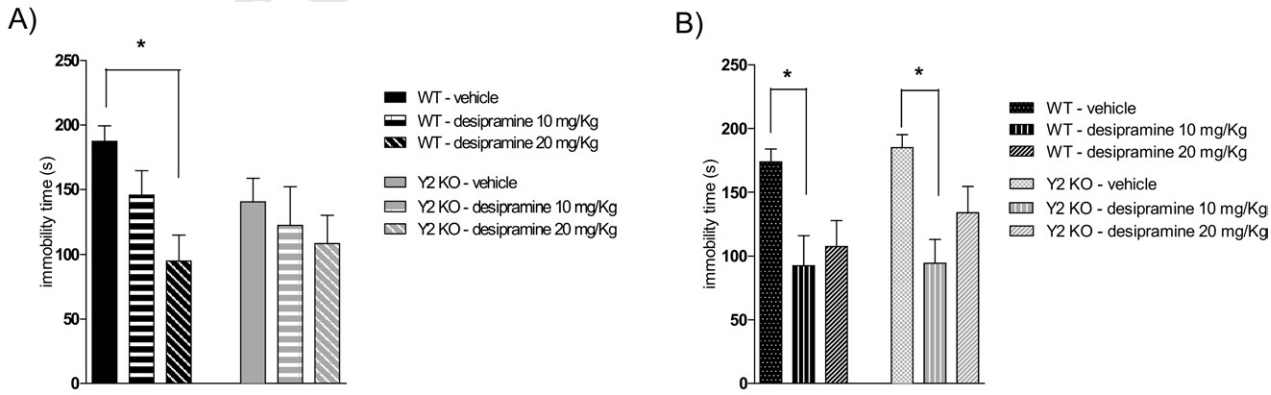

Fig. 3. Forced swim test (FST) –10 min, after desipramine treatment: the data, expressed as seconds of immobility (mean±SEM), were calculated between 2 and 6 min of test. The test was performed on: (A) WT male mice (n=8) and NPY-Y2 KO male mice (n=8), injected i.p. with vehicle, 10 mg/kg or 20 mg/kg desipramine 30 min before the beginning of the FST; * P<0.05. (B) WT female mice (n=10–11) and NPY-Y2 KO female mice (n=10–11), injected i.p. with vehicle, 10 mg/kg or 20 mg/kg desipramine 30 min before the beginning of the FST; * P<0.05.

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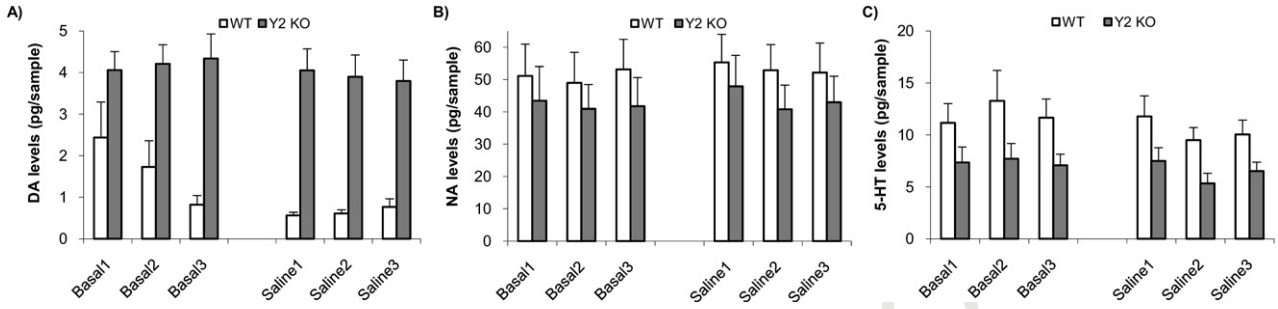

Fig. 4. Basal and post-saline injection levels of DA (A), NA (B) and 5-HT (C) in WT (white bars) and NPY-Y2 KO (gray bars) mice. Basal 1, 2, 3 and Saline 1, 2, 3 correspond to the three 20 min samples. Averaged basal DA levels were higher in the NPY-Y2 KO than in the WT mice compared to averaged post-saline levels. DA levels did not stabilize until saline injection in the WT animals (genotype×time interaction: $P<0.05$) in contrast to NPY-Y2 KO mice. Basal and saline levels did not differ significantly in WT and KO animals for both NA and 5-HT.

NPY-Y2 KO mice showed similar levels of the two hormones (ACTH: $F_{1,28}=0.5$, $P=0.49$, n.s.; corticosterone: $F_{1,28}=0.75$, $P=0.39$, n.s.). In addition, no significant effects of genotypes×stress interaction were detected (ACTH: $F_{1,28}=0.15$, $P=0.7$, n.s.; corticosterone: $F_{1,28}=0.51$, $P=0.48$, n.s.).

Microdialysis

The basal levels of DA averaged over the 1-h sequence of sampling were higher in the NPY-Y2 KO mice (3.92±0.30 pg/sample) compared with WT mice (1.66±0.37 pg/sample; $F_{1,18}=13.99$, $P=0.015$; Fig. 4A). In contrast, basal averaged levels of NA were not statistically different in the NPY-Y2 KO mice (42.04±5.10 pg/sample) compared with their WT littermates (51.06±5.36 pg/sample; $F_{1,22}=0.53$, $P=0.47$, n.s.; Fig. 4B). Finally, basal 5-HT levels were not different between NPY-Y2 KO (17.04±6.09 pg/sample) and WT animals (12.03±1.27, $F_{1,21}=0.28$, $P=0.6$, n.s.; Fig. 4C). The basal DA levels remained unstable in WT mice until the vehicle injection (genotype×time interaction; $F_{2,36}=3.49$, $P=0.04$), whereas basal NA and 5-HT levels both in NPY-Y2 KO and WT mice and DA levels in the NPY-Y2 KO mice were stable after 2 h. However, ANOVA of DA levels during the hour after saline injection revealed a significant effect of genotype ($F_{1,18}=32.55$, $P<0.001$), no effect of time ($F_{2,2}=0.12$, $P=0.88$, n.s.) and no genotype×time interaction ($F_{2,36}=2.43$, $P=0.1$, n.s.). This suggests that basal DA levels were lower in WT than in NPY-Y2 KO mice but stable for both genotypes. A similar observation was made for both basal NA and 5-HT levels. Hence, with the aim to uniform data presentation and analyses, data for basal DA, NA and 5-HT levels were all expressed as percent of the averaged levels following saline administration for both WT and NPY-Y2 KO mice and not of the real basal levels (Fig. 5).

Yohimbine administration significantly stimulated DA release in WT but not in NPY-Y2 KO mice. In the WT mice, this increase was maximal 100 min after yohimbine injection reaching 232.7±42.0% of saline. ANOVA revealed a significant effect of time ($F_{8,8}=8.21$, $P<0.001$) and a significant genotype×time interaction ($F_{8,152}=5.32$, $P<0.001$; Fig. 5A). A correlation study between basal DA levels and DA levels following yohimbine administration failed to significantly correlate them both in the WT and NPY-Y2 KO mice (data not shown).

Yohimbine administration increased NA release reaching, in WT animals a maximum of 161.9±10.2% of basal levels 40 min after injection. In NPY-Y2 KO animals, this increase (161.8±13.3% of basal levels) became maximal 60 min after injection. ANOVA applied to the post-admin-

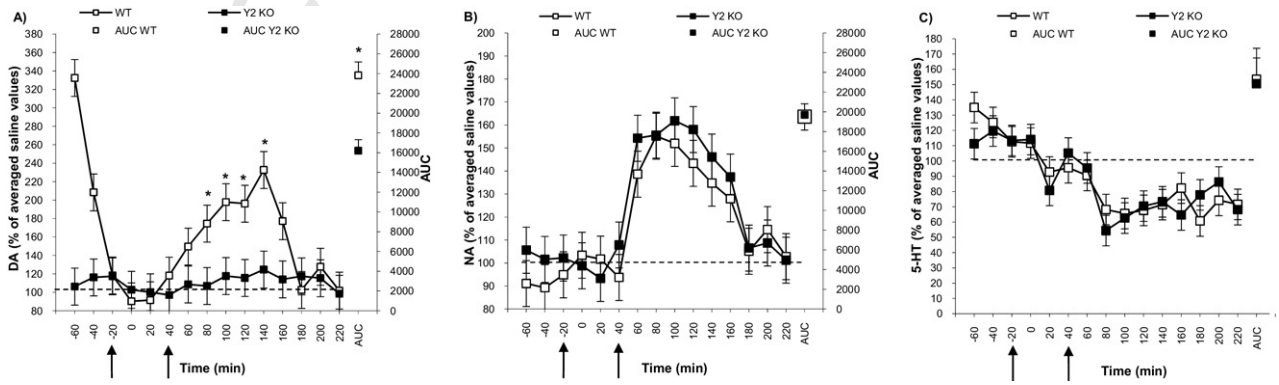

Fig. 5. Effect of yohimbine (2.5 mg/kg i.p.) on DA (A), NA (B) and 5-HT (C) levels in WT (open symbols) and NPY-Y2 KO (black symbols) mice. All results are expressed as percent of the averaged levels measured between saline (first arrow) and yohimbine administration (second arrow). * $P<0.05$ versus NPY-Y2 KO. Area under the curve (AUC, right y-axis) was calculated between 40 and 220 min with Simpson's rule on post-treatment percent-transformed data.

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istration data revealed a significant effect of time ($F_{1,8}$= 13.97, $P<0.001$) but no genotype effect ($F_{1,8}$=0.18, P=0.89, n.s.) and no genotype×time interaction ($F_{8,176}$= 0.53, P=0.83, n.s.) suggesting that the effect of yohimbine was similar in both genotypes (Fig. 5B).

Finally, 5-HT levels decreased similarly in both WT and KO mice following yohimbine treatment by 65.6±5.0%, and 53.6±7.9%, 60 min and 40 min after yohimbine injection, respectively. ANOVA of these data revealed a significant effect of time ($F_{8,8}$=2.98; P=0.038), but no effect of genotype ($F_{1,8}$=0.18, P=0.67, n.s.) or genotype×time interaction ($F_{8,160}$=1.57, P=0.13, n.s.), suggesting that this decrease was similar for both genotypes (Fig. 5C).

DISCUSSION

In the present study, NPY-Y2 KO mice backcrossed to a C57BL/6 congenic background were characterized in behavioral tests related to emotional behavior and in their neurochemical response to yohimbine administration in order to further investigate the hypothesis of the involvement of the NPY-Y2 receptor subtype in anxiety and stress-related behaviors. A general basal behavioral analysis was initially performed and the increase in basal exploratory activity found in male NPY-Y2 KO mice exposed to a new environment suggested a reduced anxiety-like state, given that the exploratory behavior is also related to a less anxious state of the animals (Fig. 2). The same analysis was performed in female NPY-Y2 KO and WT mice, but no changes were found, underlining basal gender differences. A behavioral variability between gender is not surprising; indeed it has been previously described that the emotional behavior could be influenced by endocrine factors, differing between genders (Lathe, 2004). Moreover, there could be a strong influence of gender on both baseline behavioral characteristics (Voikar et al., 2001) and antidepressant responses in different mouse strains (Caldarone et al., 2003). Indeed, looking at the behavioral response to the treatment with the antidepressant desipramine, a similar response to the pharmacological treatment was observed between female WT and NPY-Y2 KO mice (Fig. 3B), whereas a differential response was found between WT and NPY-Y2 KO male mice (Fig. 3A): in particular, the treatment with the antidepressant was not effective in NPY-Y2 KO male mice. This differential response to the antidepressant was not strictly related to NPY-Y2 deletion, since female mice respond similarly, rather to a gender difference.

Additionally, the depressive-like state of both male and female WT and NPY-Y2 KO mice was tested with FST, but no differences were found between the two genotypes in both genders. Finally, the anxiety-related behavior of male WT and NPY-Y2 KO mice was assessed with the EPM, even after an acute stress challenge, but the response observed in the different groups of animals was similar.

Moreover, in view of the role of NPY and of the potential role of NPY-Y2 receptor in hypothalamic-pituitary-adrenal axis activity, the hormonal response to stress was considered in NPY-Y2 KO and WT male mice exposed to an acute stress (FST at 21 °C) compared to non stressed mice; however, although hormonal levels were markedly up-regulated following exposure to stress, as expected (Assenmacher et al., 1995), the response was not different in the two genotypes.

These results were in contrast with previous findings in which significant behavioral differences were observed in the same NPY-Y2 KO and WT mice, in which the initial mixed 129SvJ-C57BL/6 genetic background has been maintained (Tschenett et al., 2003). The behavioral differences observed between mixed background 129SvJ-C57BL/6 mice and congenic C57BL/6 mice seemed not to be influenced by endogenous NPY hypothalamic levels, since it has been observed that pure C57BL/6 and 129SvJ mouse strains did not show basal NPY differences in this brain region. Indeed, distinct and specific differences in the anxiety-related behaviors, depressive-like state and response to stressors have been demonstrated in different mouse strains, indicating that such behaviors are influenced by genetic components (Griebel et al., 2000; Anisman et al., 2001; Belzung, 2001). The situation may be even more complicated when a genetic manipulation is also present; indeed, interactions between genetic deletions and background strains can occur and result in phenotypic differences (Carlson, 1997; Cook et al., 2002; Lesch et al., 2003). In this particular case, a variety of studies in C57BL/6 mice demonstrated higher levels of basal immobility in the FST and in the tail suspension test compared to many other strains, including the 129Sv strain (Ducottet and Belzung, 2005; Jacobson and Cryan, 2007). This hypothesis was confirmed by the results obtained in the present study with 10-min FST, in which the immobility time spent by congenic background C57BL/6 WT mice was around 280 s (data not shown), whereas the immobility time spent by the mixed background 129SvJ-C57BL/6 WT mice was around 170 s (Tschenett et al., 2003), possibly due to the presence of the 129SvJ genetic component. Moreover, other studies have demonstrated reduced or absent behavioral sensitivity to stressful stimuli, such as acute inescapable shock and chronic unpredictable stress in the C57BL/6 mouse strain (Shanks and Anisman, 1993; Mineur et al., 2003; Ducottet et al., 2004; Pothion et al., 2004; Ducottet and Belzung, 2005). The effects of stress are consistent with the present findings, in which an acute stress did not induce behavioral effects in the EPM in NPY-Y2 KO and WT male mice with pure C57BL/6 genetic background. In support of the present findings, other examples of the same genetic deletion on multiple genetic background mice led to different anxiety-related phenotypes (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998).

Differences between strain phenotypes could not only represent a reflection of the genotypes, but also of variations in the experimental methodologies and equipments performing the experiments (Crabbe et al., 1999; Crabbe and Wahlsten, 2003). The experimental conditions adopted in the present study reproduced as much as possible those described by Tschenett and colleagues. However, to exclude any possible methodological differences, a cohort of animals

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was sent to Innsbruck University, where the FST and the EPM were performed at their working conditions. Nevertheless, they exactly reproduced the results obtained in this study (data not shown), thus excluding this hypothesis. The obtained results do not anyway invalidate the previous ones in supporting a role of NPY-Y2 receptor in anxiety behaviour: indeed, it could be possible that the genetic background somehow silences this effect in the C57BL/6 strain and enhances it when expressed in the mixed background 129SvJ-C57BL/6 mice.

To further investigate the potential role of NPY-Y2 receptors depletion in these animals, the neurochemical response to a pharmacological anxiogenic challenge induced by yohimbine administration was studied. To the best of our knowledge, this is the first report investigating the neurochemical effects of yohimbine in the mPFC of mice. Nevertheless, our results resemble published observation from rats mPFC showing that yohimbine increases NA and DA efflux (Tanda et al., 1996; Garcia et al., 2004) and decreases 5-HT in naive, WT animals (Garcia et al., 2004). In addition, we demonstrated here that knocking out the NPY-Y2 receptor did not affect the increase of noradrenergic tone induced by yohimbine nor the decrease in basal 5-HT release in the mPFC. Moreover, we found that the DA release stabilized more quickly in NPY-Y2 KO than in WT mice but, only in the latter, yohimbine was able to increase DA efflux. Finally, the ability of yohimbine to increase DA release was not correlated with the DA levels preceding administration, suggesting that the inability of yohimbine to further increase DA levels in NPY-Y2 KO mice could be due to the already largely elevated levels in the basal conditions, whereas it could hardly be attributed to a ceiling effect. In our study, basal DA release was higher in the NPY-Y2 KO mice compared with WT (Fig. 4A). In contrast, both NA and 5-HT levels were unaffected by the mutation (Fig. 4B, C). These results are in line with recent findings showing that the expression of tyrosine hydroxylase, the enzyme synthesizing DA and NA in a rate limiting fashion, was increased in the brain of mice lacking the NPY-Y2 receptor (Shaw and Gehlert, 2007). Our results also suggest that the compensatory over-expression of tyrosine hydroxylase in NPY-Y2 KO mice might selectively affect DA synthesis and release without changing those of NA, a hypothesis that would warrant further investigation. These results, however seem to contradict those of Adewale et al. (2007) showing that acute administration of NPY increases DA release via NPY-Y2 receptors. The differences in both approaches including complete deletion of the NPY-Y2 receptor over the full mice development as well as the complex interplay between brain areas *in vivo* might account for the differences between our findings and those of Adewale et al.

Recent behavioral observations demonstrated that NPY-Y2 KO mice are more impulsive in the five-choice serial reaction time test than their WT littermates (Greco and Carli, 2006). DA, NA and 5-HT dynamics in both the mPFC and nucleus accumbens of the rats have been implicated in impulsive behavior in rodents (Winstanley et al., 2006). Moreover, previous observations support the contention that it is the balance between dopaminergic and noradrenergic tones that influences the degree of impulsivity as described in spontaneously hypertensive rats (Russell, 2002). In our case, while basal DA levels were higher in the NPY-Y2 KO compared with WT mice, NA levels were unchanged, hence modifying the DA/NA ratio between both basal genotypes (not shown). It can reasonably be postulated that the consequences of NPY-Y2 receptor deletion on the basal neurochemical tone could be responsible for this behavior, although in our pure C57BL/6 background mice these tests were not performed. This hypothesis would warrant further investigation.

In the present study, we used yohimbine application for its potency to induce noticeable neurochemical changes associated with its anxiogenic properties in rodents (Abercrombie et al., 1988) and to induce a strong challenge which could potentially unmask behavioral traits of these animals. In particular, an increase in rat brain NA levels and a rise in plasma concentration of NA metabolites in humans were shown to occur following yohimbine administration. By analogy in rodents, these changes were correlated with the ability of the compound to reduce the number of rats' entries in the open arm of the elevated plus maze (Johnston and File, 1989) and the self-reported anxiety in human (Charney et al., 1989), respectively. Furthermore, increase in NA efflux was found to increase in rats and mice under the influence of diverse stressing conditions including tail pinch (Page et al., 2005), forced swimming (Page et al., 2003) and exposure to novelty (Mason et al., 1998). Altogether, these observations suggest that NA turnover is, at least in part, involved in the modulation of anxiety in both rodents and human. However, in the present study, the effects of yohimbine on NA efflux were similar in both the NPY-Y2 KO and WT mice (Fig. 5B) and hence hardly can be attributed to the differential response of the animals to stressful stimuli. Modifications in DA-ergic activity have been associated with mild to sustained stressful events (Dazzi et al., 1995; Feenstra et al., 1998) and anxiogenic drug administration (Tanda et al., 1996). Our results reproduce and extend these previous observations showing that yohimbine increases DA release in the mice mPFC but that this increase is abolished in mice lacking the NPY-Y2 receptor. Noteworthy, the lack of increase of DA following yohimbine administration in the NPY-Y2 KO mice is unlikely to be related to their higher basal DA levels as for both the NPY-Y2 KO and WT mice they did not correlate with the response to the pharmacological challenge. This suggests that NPY-Y2 receptors exert a direct control on tonic as well as phasic DA neurotransmission and that this control seems to be involved in the response to stressful stimulation. Further experiments would be required to decipher this interaction in more details.

CONCLUSION

In conclusion, although the present study did not confirm the anxiolytic-like behavioral phenotype of mice lacking the NPY-Y2 receptors and cross-bred in a pure C57BL/6 genetic background, it demonstrates a role of NPY-Y2 recep-

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tor in the release of dopamine following yohimbine challenge, suggesting that NPY-Y2 could be involved in the modulation of emotional behavior. In addition, these results are consistent with the concept that genetic background may influence mood-related behavioral phenotype in mice, suggesting caution when using KO mice with mixed genetic backgrounds.

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