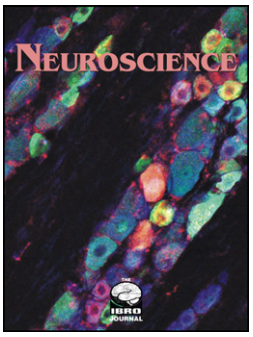

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Increased novelty-induced motor activity and reduced depression-like behavior in NPY-Y4 receptor knockout mice

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Abbreviations

AP	area postrema
CNS	central nervous system
HSD	honestly significant difference
icv	intracerebroventricular
IO	inferior olive
ip	intraperitoneal
KO	knockout
Lox	locus of cross over
lux	SI unit of illuminance and luminous emmittance
MeA	medial amygdala
NA	nucleus ambiguus
NPY	neuropeptide Y
NTS	nucleus of solitary tract
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PP	pancreatic polypeptide
PYY	peptide YY
SSRI	selective serotonin reuptake inhibitor
VMH	ventromedial hypothalamus

ABSTRACT

There is growing evidence that neuropeptide Y acting through Y1 and Y2 receptors has a prominent role in modulating anxiety- and depression-like behavior in rodents. However, a role of other Y-receptors like that of Y4 receptors in this process is poorly understood. We now investigated male Y2, Y4 single and Y2/Y4 double knockout mice in behavioral paradigms for changes in motor activity, anxiety and depression-like behavior. Motor activity was increased in Y2, Y4 and Y2/Y4 knockout mice under changing and stressful conditions, but not altered in a familiar environment. Y4 and Y2 knockout mice revealed an anxiolytic phenotype in the light/dark test, marble burying test and in stress-induced hyperthermia, and reduced depression-like behavior in the forced swim and tail suspension tests. In Y2/Y4 double knockout mice, the response in the light/dark test and in the forced swim test was further enhanced compared to Y4 and Y2 knockout mice, respectively. High levels of Y4 binding sites were observed in brain stem nuclei including nucleus of solitary tract and area postrema. Lower levels were found in the medial amygdala and hypothalamus. Peripheral administration of PP induced Y4 receptor-dependent c-Fos expression in brain stem, hypothalamus and amygdala. PP released peripherally from the pancreas in response to food intake, may act not only as a satiety signal but also modulate anxiety-related locomotion.

Keywords: pancreatic polypeptide (PP), neuropeptide Y (NPY), anxiety, depression, amygala, area postrema.

INTRODUCTION

Pancreatic polypeptide (PP) is a 36 amino acid hormone, which is predominantly synthesized in type F islet cells of the pancreas (Berglund et al., 2003). Like neuropeptide Y (NPY) and peptide YY (PYY) it belongs to the so-called PP-fold peptide family (Berglund et al., 2003; Larhammar, 1996). Several $G_{i/o}$ coupled receptors (Y1, Y2, Y4, Y5, y6) (Michel et al., 1998) are mediating actions of these peptides. Those of PP may be preferentially transduced by Y4 receptors (Berglund et al., 2001; Gehlert et al., 1996).

Whereas high Y4 mRNA concentrations are present in the peripheral tissues, they are considerably lower in the brain (Bard et al., 1995; Lundell et al., 1997). PP is released into the circulation through a cholinergic, vagus-dependent mechanism upon ingestion of food, and consecutively regulates pancreas and gastric secretion, gallbladder contraction and gastrointestinal motility (Asakawa et al., 1999). In mammalians, the distribution of the Y4 receptor has been investigated in brains of the rat and marmoset so far (Dumont et al., 1998). In the rat, Y4 receptors are primarily concentrated in the hypothalamus and in the brain stem (Fetissov et al., 2004; Lundell et al., 1997; Whitcomb et al., 1997). Data on Y4 receptor distribution in the mouse brain are not yet available.

In spite of their low abundance in the central nervous system, Y4 receptors seem to be involved in the regulation of metabolic processes. Thus, Y4 receptor knockout (KO) mice revealed signs of reduced weight gain, altered expression of gonadotropin-releasing hormone as well as increased male aggressive behavior (Asakawa et al., 2003; Sainsbury et al., 2003; Sainsbury et al., 2002b). Furthermore, NPY another member of the PP-fold peptide family that is most abundantly expressed in neurons of the central nervous system exerts strong regulatory effects in emotion-related behaviors in rodents involving Y1 and Y2 receptors. Pressure injection of NPY and of Y1 receptor agonists, produced pronounced anxiolytic and antidepressant-like effects, notably in the amygdaloid nuclei (Heilig et al.,

1993; Kask et al., 2002; Redrobe et al., 2002), whereas injection of Y2 receptor agonists into the basolateral amygdala was anxiogenic (Sajdyk et al., 2002).

We and others demonstrated that deletion of Y2 receptors results in pronounced anxiolytic and antidepressant-like effects. (Carvajal et al., 2006; Redrobe et al., 2003; Tschenett et al., 2003). Recently Painsipp *et al.* demonstrated an anxiolytic and anti-depressant-like effect of Y4 receptor deletion in female mice. (Painsipp et al., 2008).

In our present study we investigated male Y4 KO mice for changes in anxiety-like behavior in the elevated plus maze (Rodgers and Dalvi, 1997; Lister, 1987; Pellow et al., 1985)and the light/dark box (Bourin and Hascoet, 2003; Crawley and Goodwin, 1980) and depression-like behavior in the forced swim test (Lucki, 1997; Petit-Demouliere et al., 2005; Porsolt et al., 1977) and tail suspension test (Cryan et al., 2005; Steru et al., 1985). In addition, basal and novelty-induced locomotor activity were examined by homecage activity and in the open field test (Hall, 1934; Prut and Belzung, 2003), respectively. For differentiating anxiolytic-like behavior from changes in novelty-induced locomotor activity, we additionally used stress-induced hyperthermia (Borsini et al., 1989; Lecci et al., 1990; Olivier et al., 2003; Van der Heyden et al., 1997) and the marble burying test (Borsini et al., 2002; Broekkamp et al., 1986; Nicolas et al., 2006; Njung'e and Handley, 1991; Xu et al., 2004), that are independent or even inversely dependent on motor activity, respectively. In the marble burying test, selective suppression of marble burying is suggested to correlate with anxiolytic behavior (Borsini et al., 2002; Xu et al., 2004). Furthermore, for investigating whether effects of Y4 deletion are dependent or independent of Y2 receptor-mediated mechanisms, we conducted these behavioral tests concomitantly in Y2 single-KO and Y2/Y4 double-KO mice.

For identifying possible anatomical sites integrating changes in emotional motor behaviors, we studied for the first time the distribution of Y4 receptors by receptor binding autoradiography in the mouse brain. Since in this study we observed high concentrations of Y4 receptors in brain areas accessible to peripheral blood circulation, such as the area

postrema, we also investigated in wildtype and Y4 KO mice the effect of ip injection of PP on c-Fos expression in the brain.

EXPERIMENTAL PROCEDURES

Animals

All experiments were conducted with adult male mice (10-16 weeks old, weighing 25-30g) maintained on a C57BL/6-129SvJ background. They were housed in groups of 3 to 5 animals under standard laboratory conditions (12h/12h light/dark cycle, lights being on at 07:00, food and water *ad libitum*). Generation of Y2, Y4 and Y2/Y4 KO animals has been described in detail previously (Sainsbury et al., 2002a; Sainsbury et al., 2002b). In brief, chimeric conditional-knock-out floxed mice ($Y2^{lox/lox}$ or $Y4^{lox/lox}$) were crossed with oocyte-specific Cre-recombinase expressing C57BL/6 mice (Schwenk et al., 1995). Non-induced $Y2^{lox/lox}$ and $Y4^{lox/lox}$ mice were used as controls for Y2 KO and Y4 KO mice, respectively (see also below). As shown previously and verified in our present experiments, the phenotype of these controls did not differ from wild-type mice of the mixed C57BL/6-129SvJ background (Sainsbury et al., 2002a). Deletion of Y2 and Y4 receptors was confirmed in all mice used for the experiment by polymerase chain reaction (PCR) and agarose gel electrophoresis and demonstrated by *in situ* hybridization and receptor autoradiography (human (h) [^{125}I]-PYY_{3-36} for Y2 receptors, rat (r) [^{125}I]-PP for Y4 receptors) for randomly selected mice.

Genotyping

Genotypes of the mice were monitored as described previously (Sainsbury et al., 2002a). In brief, PCR was performed using the following primers for the Y2 receptor oligo-Y2C (5´ TTA ACA TCA GCT GGC CTA GC 3´), oligo-Y2D (5´GGA AGT CAC CAA CTA GAA TGG 3´), oligo-Y2E (5`AGC ATC CAG AGA AGT GCA AC 3´) and for the Y4 receptor oligo-Y4A (5` ATC CTT CCT GCC TCT ATG 3´), oligo-Y4B (5´ GGA TAA TAC CAG CAT GGC 3´), oligo-

Y4C (5`GCA TCT GTA CTG AGT GGC 3`), with 40 cycles of 94°C for 45s, 59°C for 45s and 72°C for 45s. DNA was loaded on a 2% agarose gel. Ethidium bromide labeled bands were evaluated under UV light and monitored with a concomitantly run size marker. Using a combination of oligo-Y2C and oligo-Y2D or oligo-Y4A and oligo-Y4B a floxed DNA sequence (Y2 $^{lox/lox}$ mice: band corresponding to 365 bp; Y4 $^{lox/lox}$: 210 bp) and a sequence corresponding to the intact Y2 or Y4 receptor gene could be detected (Y2 wildtype mice: 330 bp; Y4 wildtype mice: 350 bp), whereas oligo-Y2C and oligo-Y2E or oligo-Y4A and oligo-Y4C were used to demonstrate the deletion of the Y2 receptor or Y4 receptor, respectively (Y2 KO mice: 250 bp; Y4 KO mice: 290 bp); (for details see (Sainsbury et al., 2002a; Sainsbury et al., 2002b).

Behavioral experiments

Animal care and experimental protocols were approved by the Austrian Ministry of Science. Prior to behavioral testing all animals were allowed to habituate to the test room for at least 24 h. All behavioral tests (except stress-induced hyperthermia) were conduced between 08:00 and 12:00 a.m. The respective setups had been validated pharmacologically in-house before. Behavioral testing was performed in groups of 8-10 mice per genotype and always replicated in separate experiments using naïve cohorts of mice. In all cases experiments revealed the same outcome of behavioral changes. In the initial set of experiments, Y4 and Y2 KO mice were compared with their respective controls ($Y4^{lox/lox}$, $Y2^{lox/lox}$ mice, respectively are referred to as “controls”). The floxed mice ($Y4^{lox/lox}$, $Y2^{lox/lox}$) were considered as the most appropriate controls, since KO mice used for the study were derived from these strains. In the repetition experiments, Y4 and Y2/Y4, or Y2 and Y2/Y4 KO were tested against control mice. In separate experiments $Y2^{lox/lox}$ and $Y4^{lox/lox}$ mice were also compared to WT mice on the same mixed background and no difference between controls ($Y4^{lox/lox}$, $Y2^{lox/lox}$) and WT mice was observed in any of the performed tests (data not shown). All experiments were done in a blinded and randomized fashion. The genotype of all mice was verified after each experiment.

Tests of motor activity

Home cage activity. Activity measurements were performed in the home cage of mice for 60 h. Mice were single-housed in standard cages with food and water *ad libitum*. Movements of the mice were determined by an infrared sensor throughout light and dark phases (TSE LabMaster InfraMot, Bad Homburg, Germany). After a 24 h acclimatization period, cumulative activity measurements were evaluated in the subsequent 24 h period. Our setting allowed concomitant testing of four KO mice and four controls. Testing was performed during the weekend to avoid any disturbance. Data obtained at two successive weekends were pooled for final evaluation of home cage behavior.

Open field test. The open field test was conducted as previously described (Tschenett et al., 2003). The setup for the open field test consisted of four individual gray plastic boxes (50 x 50 x 30 cm) illuminated with 150 lux. Boxes were arranged in a square and two controls and two KO mice were tested simultaneously. Analysis was done using the VideoMot2 system (TSE Systems, Bad Homburg, Germany). For evaluation of motor behavior each box was virtually divided in a 34 x 34 center zone, and a surrounding 8 cm border belt. KO mice and controls were placed individually into the center of a box and their behavior was tracked for 10 min.

Tests of anxiety

Elevated plus maze. The elevated plus maze was performed as previously described (Pellow et al., 1985; Lister, 1987). The apparatus was obtained from TSE Systems (Bad Homburg, Germany). It consisted of an elevated plus (74 cm above the floor) with two opposing open arms (30 x 5 cm) and two opposing closed arms (30 x 5 x 14 cm). At the beginning mice were placed on the center platform (5 x 5 cm) facing an open arm. Illumination of the open arms was 80 lux. Testing time was 5 min and arm entry was defined when the mouse had

placed all four paws into an arm. Analysis was performed by a VideoMot2 system (TSE Systems, Bad Homburg, Germany).

Light/dark test. For the light/dark test we followed the procedure described by Crawley and Goodwin, (1980). The apparatus consisted of a dark plastic compartment covering 1/3 (16 x 50 cm) and a bright compartment covering 2/3 (34 x 50 cm) of the testing area. The two parts were connected by a central opening (7 x 7 cm) on floor level. Illumination in the light compartment was 400 lux, that of the dark compartment 1 lux. Mice were placed individually into the dark compartment facing away from the opening. The behavior of each mouse was then tracked for 10 min and analyzed by VideoMot2 system (TSE Systems, Bad Homburg, Germany).

Stress-induced hyperthermia. The test was performed between 13:30 and 15:00 h. Mice were carried to the experimental room and put individually into holding cages. After an adaptation period of 90 min basal temperature was obtained with a rectal mouse probe (Van der Heyden et al., 1997). The probe was 1.5 mm in diameter and inserted 20 mm into the mouse rectum, while the animal was gently restrained manually. Before insertion the probe was dipped into silicone crème and maintained in the rectum until stable values were obtained (20 s). Temperature was measured to the nearest 0.1 °C (Omega HH147 RS-232, Newport Electronics GmbH, Deckenpfronn, Germany). After 10 min the second value was determined to calculate the stress-induced hyperthermia ($\Delta T = T2-T1$).

Defensive marble burying. Mice were kept in the experimental room for adaptation for at least 120 min (Njung'e and Handley, 1991). The experimental setup consisted of polyethylene cages (38 x 22 x 16 cm) containing 24 glass marbles (1.5 cm diameter) evenly spaced on 5 cm deep rodent sawdust bedding. The cage was closed with an even metal grid on top of which an infrared sensor was mounted for activity measurements. No food or water was present during the 30 min observation period. At the end of the experiment all marbles

covered at least two-thirds by sawdust were determined. Motor activity was recorded using an infrared sensor (TSE LabMaster InfraMot, Bad Homburg, Germany) during the whole test.

Tests of depression-like behavior

Porsolt's forced swim test. The forced swim test was conducted as described previously (Petit-Demouliere et al., 2005; Porsolt et al., 1977). In brief, mice were placed in an open glass cylinder (diameter 12 cm, height 24 cm, water level 16 cm) containing fresh tap water of 23-25 °C. The duration of the test was six min. The last four min were analyzed in one min bins. Floating was defined when the mouse performed only those movements required to keep its head above the water surface. The test was videotaped and evaluated by two different experimenters blind to the genotype of the mice.

Tail suspension test. The tail suspension test was performed as described previously (Mayorga and Lucki, 2001; Steru et al., 1985). Mice were suspended by the tail to a bar in a box open to the camera at the front (28 x 28 x 40 cm) and the behavior was recorded for a 6 min period. The experiment was evaluated independently by two trained observers blind to the genotype of the mice. Immobility was defined as the absence of limb movement.

Histochemical method

In situ hybridization for Y2 receptors. Mice were killed by exposure to CO_2 gas. Brains were rapidly removed and frozen by immersion in -70°C isopentane (Merk, Darmastadt, Germany). Coronal 20 µm sections were cut using a cryostate-microtome (Carl Zeiss AG, Germany), and thaw-mounted on adhesive, silane-coated slides. These sections were either used for *in situ* hybridization or receptor autoradiography.

In situ hybridization of Y2 receptor mRNA was done essentially as described previously (Schwarzer et al., 1998). Two oligonucleotides complementary to nucleotides 711-762: 5´GCT CTC CAG GTG GTA GAC AAT GCA ACG ATG GCG GTC CAG AGC AAT GAC TGT

C 3´ and 1415-1458: 5´ CCA TGT GCT TTC ACA CCT GTG TCC TTA CAC ATT GGT AGC CTC CG 3´) of the mouse Y2 receptor were labeled with [^{35}S]thio-dATP by deoxynucleotidyltranferase (Roche, Mannheim, Germany). Sections were incubated concomitantly with both ^{35}S labeled oligonucleotides for 18 h and then washed stringently and exposed to Biomax films (Kodak, France).

Receptor autoradiographies for Y2 and Y4 receptors. For Y2 and Y4 receptor autoradiographies [^{125}I]hPYY$_{3-36}$ and [^{125}I]rPP were used as radioligands, respectively. Assays were performed as described previously (Furtinger et al., 2001). rPP and hPYY$_{3-36}$ were freshly radiolabeled using the chloramin-T method and purified by high performance liquid chromatography. Coronal 20 µm sections (see above) from control and Y4 KO mouse brains were preincubated in 200 ml modified Krebs-Henseleit buffer (in mM: 118 NaCl, 4.8 KCl, 1.3 $MgSO_4$, 1.2 $CaCl_2$, 1.2 KH_2PO_4, 15 $NaHCO_3$, 50 glucose 10 Tris/ HCl, pH 7.4) for 60 min at room temperature. Incubation was performed in Hellendahl jars with 50 ml of the same buffer supplemented with 0.1% bovine serum albumin and 0.05% bacitracin and 50 pM of the respective radioligand for 120 min. For termination of the reaction sections were dipped twice in ice cold Krebs-Henseleit buffer, washed 30 sec in the same buffer, dipped in de-ionized ice cold water and rapidly dried under a stream of cold air. The slides labeled with [^{125}I]hPYY$_{3-36}$ and [^{125}I]rPP were then exposed to Biomax films (Kodak, France) for 7 and 21 days, respectively. Unspecific binding was determined by incubating subsequent sections in the presence of 1 µM NPY.

c-Fos expression in the amygdala after peripherally applied PP

Injections of PP. Twelve wild type and eight Y4 KO mice, fasted over night, were injected i.p. with PP (1.0 mg/kg) or vehicle (phosphate buffered saline) in a volume of 1 ml/kg between 10:00 to 12:00 h. At 30 min and 90 min after ip injection mice were deeply anaesthetized with ketamine / xylazine (100 mg/kg and 20 mg/kg from Parke Davis-Pfizer, Sydney, Australia and Bayer AG, Leverkusen, Germany, respectively) and perfused via the left ventricle with

25 ml phosphate buffered saline following by ice-cold 4% paraformaldehyde in phosphate buffered saline (PBS). Brains were immediately removed and placed in 4% paraformaldehyde for 30 min then in PBS containing 30% sucrose overnight.

c-Fos immunohistochemistry. Coronal slices of 30 µm thickness were mounted on slides and washed in 1% H_2O_2 in 50% alcohol for 20 min to abolish endogenous peroxidase activity. Sections were incubated overnight at room temperature with the primary antibody, rabbit-anti-mouse c-Fos (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), diluted at 1:4000 in phosphate buffered saline containing 0.1% Triton x-100. After three 10-min washes in PBS-Triton, sections were incubated with the biotinylated secondary anti-rabbit antibody (Sigma-Aldrich, St Louis, MO, USA), diluted 1:250 in PBS for 3 h. Sections were washed three times for 10 min each in PBS and then incubated with Avidin-Biotin-Peroxidase Vectastain® (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. Sections were rinsed in PBS and treated with diaminobenzidine (Dako, Carpinteria, CA, USA) for 5 min. Slides were rinsed in water and dehydrated by passing them through increasing concentrations of ethanol and then in xylene before mounting.

Quantification of c-Fos positive neurons. Sections were screened for visualization of c-Fos-like immunoreactivity (Fos-IR) neurons using an Axiophot microscope. In regions differences in Fos-IR neurons were readily apparent, Fos-positive nuclei were counted following the area outline. Every third section was counted for each nucleus for quantification of Fos-IR neurons. According to the atlas of mouse brain in stereotaxic coordinates by Franklin and Paxinos (Paxinos and Franklin, 2001). The value represents an average of Fos-IR neurons cells were based on relative size and dark stained nuclei. Comparisons were made between two groups stained at same time. The average of cell counts in each nucleus was determined from both left and right sides, and then all groups were pooled for final analysis.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (Prism 4 for MacIntosh, GraphPad Software Inc., San Diego, CA). Effects of genotype was assessed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test for *post hoc* analysis of group differences. Comparison of two groups was done by Student's T test. All data for the analysis of c-Fos expression in the brain were assessed by factorial ANOVA followed by Fisher's or Contrasts *post hoc* tests, using StatView version 4.5 or Super-ANOVA (Abacus Concepts Inc, CA, USA). Group data are expressed as mean $\pm$ S.E.M. Statistical significance was set at $P<0.05$.

RESULTS

Distribution of Y2 and Y4 receptors

In situ hybridization and receptor autoradiography revealed high levels of Y2 receptor mRNA and binding sites in various limbic brain areas, notably in the amygdala and the hippocampus, as previously described (Dumont et al., 1998; Gackenheimer et al., 2001; Parker and Herzog, 1999). In Y2 and Y2/Y4 KO mice Y2 receptor was absent on mRNA and protein level (data not shown). As shown in Figure 1, Y4 receptor binding was particularly enriched in different brain stem nuclei, like the nucleus of solitary tract, nucleus ambiguous (Figure 1 A), facial nucleus, area postrema and the inferior olive (Figure 1 B). Lower levels of Y4 receptors were present in the medial amygdala and in the ventromedial hypothalamus (Figure 1 F). The specificity of this binding was supported by the fact, that no signal was detected on sections from Y4 and Y2/Y4 KO mice (Figure 1 C).

Motor activity tests

Home cage activity. In order to assess general activity of Y2, Y4 and Y2/Y4 receptor KO mice, we determined the basal locomotion in the home cage. As shown in Figure 2 there was no effect of genotype in cumulative home cage activity measured during 24 h (activity counts in thousands: Y2 KO: 20.85 ± 2.28, control: 20.45 ± 2.16, P=0.91; Y4 KO: 16.52 ± 3.39, control: 17.26 ± 1.87, P=0.85; Y2/Y4 KO: 16.52 ± 2.90, control: 18.39 ± 1.52, P=0.58, n=8 /group) In contrast, Y4 KO mice displayed a higher activity during the dark phase of the first 24 h, when compared to control animals. In Y2/Y4 receptor KO mice, a second activity peak was observed before the onset of the light phase.

Open field test. The explorative drive and motor behavior in a novel environment was investigated in the open field paradigm (Figure 3). Total distance, number of center entries and number of rearings were taken as parameters of activity in an unfamiliar, potentially aversive environment. Statistical analysis by one way ANOVA indicated a genotype-related

difference in total distance ($F_{2,52}$=34.75, *P*<0.0001 for controls vs. Y2 and Y2/Y4 KO mice; $F_{2,29}$=22.66, *P*<0.0001 for controls vs. Y4 and Y2/Y4 KO mice), number of center entries ($F_{2,52}$=18.75, *P*<0.0001 for controls vs. Y2 and Y2/Y4 KO mice; $F_{2,29}$=34.15, *P*<0.0001 for controls vs. Y4 and Y2/Y4 KO mice) and vertical activity ($F_{2,52}$=3.42, *P*=0.0002 for controls vs. Y2 and Y2/Y4 KO mice; $F_{2,29}$=7.19, *P*=0.0028 for controls vs. Y4 and Y2/Y4 KO mice). *Post-hoc* analysis of the data revealed that Y2, Y4 and Y2/Y4 KO mice displayed increased explorative drive. Similarly all three groups of KO mice spent a significantly increased time in the center of the arena compared to controls, as revealed by ANOVA ($F_{2,53}$=6.55, *P*<0.0001 for controls vs. Y2 and Y2/Y4 KO mice; $F_{2,29}$=14.00, *P*<0.0001 for controls vs. Y4 and Y2/Y4 KO mice) and *post-hoc* test.

Anxiety- and depression-related behaviors

Light/dark test. The light/dark test of anxiety indicated anxiolytic phenotypes for all three knockout lines, Y2, Y4 and Y2/Y4 receptor KO mice (Figure 4). All three groups of receptor KO mice showed an increased percentage of time spent in the light compartment ($F_{2,23}$=7.50, *P*=0.0029 for controls vs. Y2 and Y2/Y4 KO mice; $F_{2,23}$=9.75, *P*=0.0008 for controls vs. Y4 and Y2/Y4 KO mice). *Post hoc* analysis revealed for Y4 KO mice (*P*<0.05) and Y2/Y4 KO (*P*<0.001) an increased time spent in the light compartment compared to controls. Also the time spent in the light box by Y2/Y4 double KO (*P*<0.05) was increased compared to Y4 KO mice. The number of transitions was higher ($F_{2,23}$=6.90, *P*=0.0029 for controls vs. Y2 and Y2/Y4 KO mice; $F_{2,23}$=7.56, *P*=0.0028 for controls vs. Y4 and Y2/Y4 KO mice), when compared to controls. The distance traveled in the light compartment (Figure 4 B) was increased in all three groups of KO mice ($F_{2,23}$=19.45, *P*<0.0001 for controls vs. Y2 and Y2/Y4 KO mice; $F_{2,23}$=16.19, *P*<0.0001 for controls vs. Y4 and Y2/Y4 KO mice), whereas no difference was observed analyzing the distance traveled in the dark compartment (Figure 4 C) ($F_{2,23}$=1.45, *P*=0.25 for controls vs. Y2 and Y2/Y4 KO mice; $F_{2,23}$=1.29, *P*=0.29 for controls vs. Y4 and Y2/Y4 KO mice).

Elevated plus maze. In the elevated plus maze (Figure 5), Y2 and Y2/Y4 KO mice spent more time on the open arms ($F_{2,54}$=10.41, *P*=0.0001 for controls vs. Y2 and Y2/Y4 KO mice; $F_{2,45}$=13.25, *P*<0.0001 for controls vs. Y4 and Y2/Y4 KO mice) and showed a higher percentage of open arm entries compared to control animals ($F_{2,54}$=5.92, *P*=0.0043 for controls vs. Y2 and Y2/Y4 KO mice; $F_{2,45}$=10.58, *P*=0.0002 for controls vs. Y4 and Y2/Y4 KO mice), as indicated by *post-hoc* analysis. Y2 and Y2/Y4 KO mice, but not Y4 KO mice revealed an increased number of closed arm entries ($F_{2,54}$=6.68, *P*=0.0023 for controls vs. Y2 and Y2/Y4 KO mice). The total distance traveled was, however, increased in all three KO lines ($F_{2,54}$=11.63, *P*<0.0001 for controls vs. Y2, and Y2/Y4 KO mice; $F_{2,45}$=16.52, *P*<0.0001 for controls vs. Y4 and Y2/Y4 KO mice).

Stress-induced hyperthermia. To obtain anxiety related parameters that are independent of motor activity we performed stress-induced hyperthermia in Y2 KO, Y4 KO, Y2/Y4 KO and control mice. Stress induced by the method *per se* (measurement of rectal temperature) lead to a significant increase in temperature (∆T). One-way ANOVA revealed a genotype related difference in ∆T ($F_{2,39}$=7.34, *P*=0.002). *Post hoc* test showed that Y4 KO (P<0.01) but not Y2/Y4 KO (*P*>0.05) exhibited a reduced ∆T compared to controls (Figure 6 A). At the same time, a significant increase of basal temperature was observed in Y4 KO (38.5 ± 0.17 °C) compared to Y2/Y4 KO (37.6 ± 0.27 °C) and wild type controls (37.6 ± 0.18 °C) as shown by one-way ANOVA ($F_{2,39}$=6.53, *P*=0.0036) and *post hoc* test (*P*<0.01 for Y4 KO vs. controls and *P*<0.01 for Y4 KO vs. Y2/Y4 KO). Similarly, Y2 KO (Figure 6 B) mice displayed a significant reduction of ∆T compared to control mice (controls: 1.2 ± 0.10 °C, Y2 KO: 0.8 ± 0.16 °C; *P*=0.02). There was no difference in basal temperature between Y2 KO (37.3 ± 0.21 °C) and control (37.5 ± 0.20 °C) mice .

Marble burying. Enhanced exploration in approach avoidance tests could be secondary to increased general activity. For investigating this aspect, we performed a marble burying test, in which reduced anxiety correlates with a decreased number of marbles buried by the

mouse in the bedding of the cage. As shown in Figure 7 A, Y4 KO mice buried significantly less marbles than control mice (controls: 9.75 ± 2.25, Y4 KO: 3.00 ± 1.60; P=0.028). Assessment of motor activity revealed increased activity of Y4 KO mice during the first part of the test compared to controls. Analysis of variance indicated that this effect was dependent on genotype ($F_{1,70}$= 6.35) and that there was a significant interaction between genotype and time ($F_{6,70}$= 2.34). The difference between genotype was apparent after 10 and 15 min (5-10 min: P<0.05; 10-15 min: P<0.01) as determined by Bonferroni *post hoc* test. Similarly, Y2/Y4 KO (Figure 7 B) buried significantly less marbles during the 30 min testing period (controls: 16.13 ± 2.07; Y2/Y4 KO: 6.50 ± 2.11, P=0.006). No difference was observed in general motor activity between Y2/Y4 KO and control mice in this test. The known anxiolytic phenotype of Y2 KO mice was confirmed in the marble-burying test (Figure 7 C) (controls: 13.50 ± 2.56, Y2 KO: 6.63 ± 1.89, P=0.048). There was no difference in general activity during the 30 min testing period between Y2 KO and controls.

Forced swim test and tail-suspension test. In order to investigate depression-like behavior in a stressful and inescapable situation we performed Porsolt's forced swim test and the tail suspension test (Figure 8). In the forced swim test, a significant decrease in floating time was apparent in Y4, Y2 and Y2/Y4 receptor KO mice, as revealed by one-way ANOVA followed by *post-hoc* analysis ($F_{2,25}$=27.81, P<0.0001 for controls versus Y2 and Y2/Y4 KO mice; $F_{2,29}$=68.42, P<0.0001 for controls vs. Y4 KO and Y2/Y4 double KO mice). In addition, *post hoc* analysis showed a lower immobility time of Y2/Y4 KO compared to Y2 single KO mice (Figure 8 A). The tail-suspension test revealed comparable effects on stress-coping ability after Y4 and Y2/Y4 receptor deletion as the forced swim test. During the 6 min testing period, Y4 KO, Y2 KO and Y2/Y4 double-KO mice spent a significantly reduced time in immobility ($F_{2,25}$=8.53, P=0.0014 for controls vs. Y2 KO and Y2/Y4 double KO mice; $F_{2,29}$=15.70, P<0.0001 for controls vs. Y4 KO and Y2/Y4 double KO mice).

Y4 receptor mediated activation of amygdala neurons. To investigate a possible influence of peripherally released PP on central mechanisms integrating emotion-related behavior, we used i.p. injection of the endogenous Y4 preferring ligand pancreatic polypeptide and analyzed the anatomical distribution of expression of the early neuronal activation marker c-Fos in the brain. Brains were isolated 30 min and 90 min after ip PP for identification of directly and indirectly activated brain regions, respectively and processed for immunohistochemistry. In addition to strong c-Fos expression (Table 1) in the area postrema there was a significant increase in c-Fos activation specifically in the arcuate nucleus, nucleus of solitary tract and medial amygdala compared to saline injected control mice 30 min after ip injection of PP (Figure 9 A and B). Expression was more pronounced in the paraventricular nucleus, in the ventromedial hypothalamus and in the lateral hypothalamic area after 90 min than after 30 min. (Table 1). Consistent with a Y4 specific action no increase in c-Fos immunoreactivity can be seen in these same areas of i.p. PP injected Y4 knockout mice (Figure 9 C and D).

DISCUSSION

Our experiments using behavioral testing in male mice from three different KO lines revealed two major changes in phenotype related to Y4 receptor deletion: 1) increased motor activity under stressful and novel conditions and 2) changes in emotional behavior as indicated by reduced depression-like behavior and in anxiety-related behavior in the light/dark test, marble-burying test and stress-induced hyperthermia (but not elevated plus maze). In several instances, emotion-related behavioral changes were more pronounced in the Y2/Y4 double KO mice than in Y4 or Y2 KO mice, suggesting that Y4 mediated mechanisms in integrating emotional behavior are coordinated with Y2 receptor activity.

Novelty-induced motor activity

Motor activity was investigated 1) under baseline, non-aversive conditions by assessing home cage activity during three consecutive light/dark cycles and 2) in a potentially stressful environment using the novel open field test. In the familiar environment, such as the home cage, there was no significant difference in general motor activity between any genotypes. The increased activities displayed by Y4 KO mice during the first dark phase, and the second peak of activity at the onset of the light phase in Y2/Y4 double KO mice, presumably reflect increased arousal responses induced by the change in conditions. These behavioral aspects were confirmed by investigating the mice in a novel open field, where Y4, Y2 and Y2/Y4 KO mice revealed increased horizontal and vertical activity compared with controls.

Anxiety-related behavior

Whereas Y2 knockout mice and Y2/Y4 double knockout mice revealed an anxiolytic phenotype in all tests (except stress-induced hyperthermia), evidence towards reduced anxiety in Y4 KO mice was ambiguous. Y4 KO mice showed less anxiety-like behavior in the light/dark and marble burying test, but not in the elevated plus maze. The additional increase in time spent and distance traveled by Y2/Y4 KO mice in the lit compartment of the light/dark

test compared to Y4 and Y2 KO mice, respectively suggests the involvement of coordinated mechanisms mediated by Y2 and Y4 receptors during emotional challenge. Most importantly, all three KO lines showed a longer total distance traveled in the elevated plus maze, as well as an increased distance traveled in the lit compartment of the light/dark box. Since motor activity was not increased in the dark compartment of the light/dark box, this phenomenon seems to be dependent on stressful novel conditions.

For further investigating the contribution of novelty-induced motor activity in the exploration/avoidance tasks, we investigated stress-induced hyperthermia test in Y4, Y2/Y4 and Y2 KO mice an anxiety test that is independent of motor behavior. This test seems to have face, construct and predictive validity in modeling anxiety related autonomous responses (Olivier et al., 2003). The observed reduction in the hyperthermic response (ΔT) points towards reduced anxiety in Y4 and Y2 KO mice. However, since the basal temperature was higher in Y4 KO mice than in WT and Y2/Y4 double KO, we cannot exclude a ceiling effect in the body temperature of Y4 KO mice. In addition, hypothalamic structures involved in temperature control are directly or indirectly targeted by NPY and PP (Parker and Herzog, 1999). Temperature control is a multi-factorial phenomenon. Therefore the implication of the stress-induced hyperthermia data may be limited. Interestingly, there was no difference in stress-induced body temperature in Y2/Y4 KO mice compared to controls. However, while basal temperature is unaltered in Y2/Y4 KO mice, there is evidence for facultative thermogenesis in this mouse strain (Sainsbury et al., 2003; Sainsbury et al., 2006). Together with altered sympathetic signaling, increased thyrotropin releasing hormone mRNA levels in the paraventricular nucleus of the hypothalamus could be a cause for changes in thermoregulation in Y2/Y4 KO mice (Ribeiro et al., 2001; Sainsbury et al., 2003; Sainsbury et al., 2006; Silva and Larsen, 1983).

For investigating further the possible dependence of emotion-related behavior on locomotion, we performed the marble burying test that is *inversely* dependent on motor activity. In this

test, selective suppression in burying marbles (considered by the mice to be potentially aversive) is investigated as a measure of anxiety-like behavior (Njung'e and Handley, 1991). In contrast to other paradigms, this test is not based on increased exploration as an index of anxiolytic-like effects (Cryan and Holmes, 2005). Predictive validity of this alternative behavioral test was obtained by the fact that both benzodiazepines, and selective serotonin reuptake inhibitors are able to reduce the number of marbles buried (Borsini et al., 2002). Moreover, the marble burying test has been applied successfully in evaluating the anxiolytic property of compounds, which increase locomotion at the same time (Xu et al., 2004). Results from this test indicate both, novelty-induced hyperlocomotion and reduced anxiety-related behavior in Y4 receptor KO mice. Similar as in the open field test, also in this test Y4 KO mice revealed *increased* general motor activity during the first 15 min. Nevertheless the number of marbles buried was *reduced* in Y4 KO mice. This test points to independent mechanisms leading to changes in novelty-induced locomotion and emotional behavior after Y4 receptor deletion.

Reduced depression-like behavior

For investigating depression-like behavior in the mice, we performed the tail suspension and forced swim tests, both models based on paradigms testing the ability of the mice to cope with stressful conditions. These tests have been pharmacologically validated to be sensitive to treatment with antidepressant drugs as well as for phenotypic changes in transgenic mice (Cryan et al., 2002; Cryan and Mombereau, 2004). Significantly reduced immobility in both tests as observed in all three KO mouse lines, mimics effects of antidepressant treatment and therefore indicates reduced depression-like behavior. Although both tests are quite similar in design, they seem to differ in biological changes underling the observed behavior. In contrast to the traditional forced swim test, the tail suspension test detects the efficacy of a broad spectrum of antidepressant treatments (Cryan et al., 2002; Lucki, 1997), including SSRIs and it is independent of swimming ability or thermoregulation (Cryan and Mombereau,

2004; Cryan et al., 2005). Since both tests revealed profound suppression of immobility, we suppose that deletion of Y4, Y2 and Y2/Y4 receptors produce a stable antidepressant effect. Changes in Y2 KO mice were even more pronounced after combined deletion of Y2 and Y4 receptors, indicating coordinated mechanisms mediated by the two receptors in reducing depression-like behavior. Changes in depression-related behavior are independent of the influence of sexual hormones since we observed similar changes also in female mice (Painsipp et al., 2008). It is interesting to note, that female Y4 KO mice revealed a clear anxiolytic effect on the elevated plus maze.

Considerations on central Y4 receptors mediating actions of peripherally released PP

Our experiments indicate that in wild-type mice Y4 receptors mediate depressant- and anxiogenic-like behavior and reduced motor activity, tightly linked to novel, unfamiliar conditions. This is supported by a recent report suggesting an anxiogenic phenotype in PP over-expressing mice (Ueno et al., 2007). The effects of Y4 receptors seem to be similarly potent as those mediated by Y2 receptors and, since they are partially additive, they appear to be coordinated with Y2 receptor-mediated mechanisms. Both, Y2 and Y4 receptors predominantly interact with Gi and Gq resulting in inhibition of cAMP accumulation (Freitag et al., 1995; Voisin et al., 2000; Walker et al., 1997). Local injections of NPY agonists (Kask et al., 2002) and site-specific deletion of Y2 receptors in amygdaloid nuclei performed by our group demonstrated an important role of the amygdala in Y1 and Y2 receptor mediated mechanisms of NPY on emotional behavior (Tasan et al., 2007). Y1 receptors mediate their action including anxiolysis primarily at postsynaptic sites, whereas Y2 receptors are mostly located presynaptically on NPY/ GABA neurons or at terminals of glutaminergic axons (Parker and Balasubramaniam, 2008), where they inhibit the release of classical transmitters (Adewale et al., 2005; Chen et al., 1997; Greber et al., 1994). The site and mode of actions through Y4 receptors is not clear yet. Taken the similar behavioral effects of Y2 and Y4 receptor deletion and the similar signaling pathways used by Y2 and Y4 receptors it may be possible that Y4 receptors, either activated by NPY or by PP, may also be located

presynaptically on terminals in brain stem or hypothalamic nuclei, resulting in an inhibition of transmitter release (Acuna-Goycolea et al., 2005). Interestingly, Y4 receptors have been also found in human astrocyte cultures, indicating a possible role of glial cells in Y4 receptor actions (Abounader et al., 1999).

Our present receptor autoradiographic studies revealed low, but significant concentrations of Y4 receptor binding in the amygdala and in the ventromedial hypothalamus. Also Y4 mRNA was observed in these areas of the rat indicating that the receptors may be expressed in local neurons (Parker and Herzog, 1999; Trinh et al., 1996; Whitcomb et al., 1990). All these observations are compatible with Y4 receptor-mediated mechanisms integrating emotional behavior in these brain areas. Furthermore, we found considerably higher levels of Y4 receptors in brain stem nuclei, like the nucleus tractus solitarius, nucleus ambiguous, the inferior olive and in the area postrema, that are mainly involved in the regulation of autonomous functions and in the vegetative response to emotions.

Studies in cell lines expressing recombinant Y4 receptors indicate that PP may be the primary PP-fold peptide acting on Y4 receptors (Berglund et al., 2001; Gehlert et al., 1996). PP is there about 100 times more potent than NPY or PYY in inhibiting binding of [^{125}I]Leu^{31}Pro^{34}PYY and [^{125}I]PP to Y4 binding sites (Gehlert et al., 1997; Yan et al., 1996). Nevertheless affinities of NPY and PYY (kB = 1 nM) are still in a physiological/pharmacological range and affinities of PP-fold peptides to Y4 receptors may be different in brain tissue (DiMaggio et al., 1985). Among the PP-fold peptides, only NPY is expressed in sufficiently high concentrations. NPY is thought to act primarily through Y1, Y2 and Y5 receptors to which it binds at affinities of 20 to 100 pM and exerts its prominent effects on anxiety and stress-coping behavior in rats and mice (Heilig, 2004). In spite of its apparently relatively low affinity to Y4 receptors, NPY appears to be the only reasonable candidate as a ligand for central Y4 receptors and NPY can still activate Y4 receptors at nanomolar concentration (Bard et al., 1995; McTigue and Rogers, 1995).

On the other hand, *in vivo*, peripheral PP is able to access PP-binding receptors in the area postrema, which circumvents the blood–brain barrier (Whitcomb et al., 1990), and a central action of PP has been established after local injection (McTigue and Rogers, 1995). Radiolabeled PP accumulates presumably at the high abundant Y4 receptors in the area postrema (Dumont et al., 2007). Thus PP released upon ingestion of food by vagal stimulation from pancreatic islets of Langhans into the circulation may be a regulator of emotional behavior by primarily acting on central Y4 receptors in areas outside the blood-brain barrier.

An earlier study reported no difference regarding anxiety behavior after single injections of mPP into the ventricles (Asakawa et al., 1999). In contrast, repeated intraperitoneal injection of mouse PP in fatty lizer Shoinogy *ob/ob* obese mice, however, resulted in an anxiolytic effect (Asakawa et al., 2003). This is in contrast to the anxiogenic effect seen in PP over-expressing mice (Ueno et al., 2007), supporting the anxiolytic effect of Y4 receptor deletion seen in the light/dark test in our present study. Differences in injection protocols (acute versus chronic, icv. versus i.p.) and possible compensational effects or location of the transgene(s) in PP over-expressing mice may explain differing observations, but leave the site and mechanisms of Y4-receptor mediated effects on emotional behavior still open.

Consistent with this is the observation made in our present study where ip administration of PP specifically increased c-Fos expression in addition to brain stem nuclei and arcuate nucleus also in the medial amygdala. These experiments indicate that also peripherally applied PP (acting e.g. in the area postrema or in the lateral hypothalamus upon Y4 receptors) may indirectly result in an activation of the medial amygdala and other nuclei related to integration of emotions, linking induction of satiety with potential changes in anxiety-related behavior (Ebner et al., 2004). The regionally different time course of c-Fos expression in different brain nuclei suggests activation of different pathways by PP treatment.

At the same time, NPY release in the medial amygdala could exert synergistic effects upon Y4 receptors present in this brain nucleus. Interestingly, lesions of the area postrema, containing high concentrations of Y4 receptors, results in increased NPY expression in the amygdala and reduced anxiety (Miller et al., 2002). Although the exact relation of the nucleus tractus solitarius with anxiety is not established, it is interesting to note that different anxiogenic drugs can activate c-Fos expression in this brain area (Singewald and Sharp, 2000). There exists also an interplay between the nucleus tractus solitarius containing high concentrations of Y4 receptors with the central nucleus of the amygdala, since it receives a dense GABA-ergic input from this brain area (Saha et al., 2000). Y4 receptors in the solitary nucleus are therefore ideally positioned for influencing GABA-ergic signals from the amygdala complex, the main structure of emotional behavior. Intriguing are also the possible roles of Y4 receptors in vagal nuclei of the brain stem where they are highly enriched. These nuclei are crucially involved in the regulation of autonomous functions presumably including the vagally regulated release of PP in the periphery.

CONCLUSION

Our data show an increase in novelty-induced locomotion and reduced depression-related behavior in male Y4 receptor knock out mice. The effects of Y4 and Y2 receptor deletion on emotional behavior may be additive as suggested by the phenotype of Y2/Y4 double KO mice. High concentrations of Y4 receptors in mouse brain areas, like the nucleus of solitary tract or the area postrema suggests an involvement of Y4 receptors in the modulation of emotionally driven autonomic responses, which is likely to influence the final step of emotional output.

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Legends to figures

Fig. 1. Autoradiographs of [^{125}I]rPP binding in coronal sections of the mouse brain. High levels of PP binding sites were observed in the nucleus of solitary tract (NTS), nucleus ambiguus (NA), the inferior olive (IO) and the area postrema (AP) (A+B), PP binding sites seen in Y4 KO mice (C), moderate levels of Y4 receptors were observed in the posteriordorsal division of the medial amygdala (MeA) and in the ventromedial hypothalamus (VMH) (F), in panels D and E Nissl stained sections adjacent to those in A and F are depicted. Scale bars: 1mm.

Fig. 2. Home cage activity of Y4 KO, Y2 KO and Y2/Y4 double-KO mice compared with Y2$^{lox/lox}$ and Y4$^{lox/lox}$ mice as controls, (A) there is no difference in home cage activity between Y4 KO and Y4$^{lox/lox}$ mice in cumulative 24 h analysis of home cage activity, although increased motor activity was observed during the dark phase of the first 24 h (arrow), (B) no difference between Y2/Y4 double KO and Y2$^{lox/lox}$ mice was apparent in cumulative 24 h home cage activity, but an increased agitation was seen during and shortly before the onset of light (arrows), (C) Y2 KO and Y2$^{lox/lox}$ mice displayed identical home cage activity (number of animals per group: 8).

Fig. 3. Open field test. (A) The time spent in the center of the arena was significantly increased in Y2 KO and Y4 KO and Y2/Y4 double KO mice, (B) higher number of center entries and (C) increased distance traveled was seen under stressful conditions in all three genotypes (Y2 KO, Y4 KO and Y2/Y4 double-KO) when compared to control. Analysis was done by one-way ANOVA and Newman-Keuls *post hoc* test. *P<0.05, **P<0.01, ***P<0.001 vs. controls, (n = 8 to 10 mice per genotype); ^{c}P<0.001 after pooling Y2/Y4 KO mice and controls from both experiments, (n=12-16 mice per genotype).

Fig. 4. Light/dark test. (A) In the light/dark test Y4 KO, Y2 KO and Y2/Y4 double KO mice spent significantly more time in the light compartment compared to controls and Y2/Y4 KO

spent significantly more time in the light compartment than Y4 KO, (B) Y4 KO, Y2 KO and Y2/Y4 KO mice had a higher distance traveled in the light compartment than controls, and Y2/Y4 KO showed a higher light distance than Y2 KO mice, (C) there was no difference in dark distance in any genotype. One-way ANOVA and Newman-Keuls *post hoc* test, **P*<0.05, ***P*<0.01, ****P*<0.001 significantly different vs. controls, $^{\#}P$<0.05, $^{\#\#}P$<0.01 significantly different vs. single KO mice (n = 10 to 15 mice per genotype).

Fig. 5. Elevated plus maze. (A) In the elevated plus maze Y2 KO and Y2/Y4 double-KO spent more time on the open arms compared to controls, (B) increased percentage of open arm entries in Y2 and Y2/Y4 KO mice compared to controls and (C) higher number of closed arm entries in Y2 KO mice compared to controls and Y2/Y4 KO mice compared to Y4 KO and controls, (D) increased total distance traveled in Y2, Y4 and Y2/Y4 KO mice. Analysis was done by one-way ANOVA and Newman-Keuls *post hoc* test. **P*<0.05, ***P*<0.01, ****P*<0.001 significantly different vs. controls, $^{\#}P$<0.05, $^{\#\#\#}P$<0.001 significantly different vs. Y4 KO (n = 14 to 19 mice per genotype) ^{a}P<0.05 (significance only after pooling controls and Y2/Y4 mice from both experiments).

Fig. 6. Stress-induced hyperthermia. (A) Reduction in stress-induced hyperthermia in Y4 KO mice compared to controls and (B) reduced stress-induced hyperthermia in Y2 KO mice. **P*<0.05, ***P*<0.01, significantly different vs. controls (n = 12 to 15 per genotype).

Fig. 7. Defensive marble burying. (A) Reduced number of marbles buried and increased activity in the first 15 min of 30 min testing period in Y4 KO mice, (B) reduction of number of marbles buried and unaltered activity in Y2/Y4 double KO mice, (C) decreased number of marbles buried in Y2 KO mice and no change in activity compared to controls. **P*<0.05, ***P*<0.01 significantly different vs. controls, Student´s *t*-test for number of marbles buried, two-way ANOVA and Bonferoni *post hoc* test for activity counts (n=8 per genotype).

Fig. 8. Porsolt forced swim test and tail suspension test. (A and B) In the forced swim test the time spent floating was significantly reduced in Y2 KO, Y4 KO and Y2/Y4 double KO mice. (C and D) Similarly, Y2 KO, Y4 KO and Y2/Y4 double KO show a significant reduction in immobility time in the tail suspension test, when compared to controls. Analysis was done by one-way ANOVA and Newman-Keuls *post hoc* test. **P*<0.05, ***P*<0.01, ****P*<0.001 significantly different vs. control, $^{\#\#}P$<0.001 different from Y2 KO, (n = 8 to 10 mice per genotype).

Fig. 9. c-Fos activation in the mouse brain after i.p. injection of PP (1.0 mg/kg). (A) High levels of c-Fos positive neurons could be found in the medial amygdala area 30 min after systemic PP administration and only background staining is observed in (B) saline injected control mice or (C) PP injected Y4 receptor knockout mice. (D) Statistical analysis after counting positive neurons in this area. Scale bar: 40 µm. N=4-6 mice, * p<0.001

Table 1. Relative change in c-Fos immunoreactivity in regions of the brain at 30 and 90 min after i.p. injection of PP in mice (1.0 mg/kg)

	30 min	90 min
ARC	++	+
PVN	+	+++
VMHDM	++	+++
LHA	+	++
NTS	+++	+
AP	+++	+
MeA	+++	+

ARC, arcuate nucleus of the hypothalamus; PVN, paraventricular nucleus of the hypothalamus; VMHDM, ventromedial hypothalamus dorsomedial aspect; LHA, lateral hypothalamic area; NTS, nucleus tractus solitarus; AP, area postrema.

Figure 1

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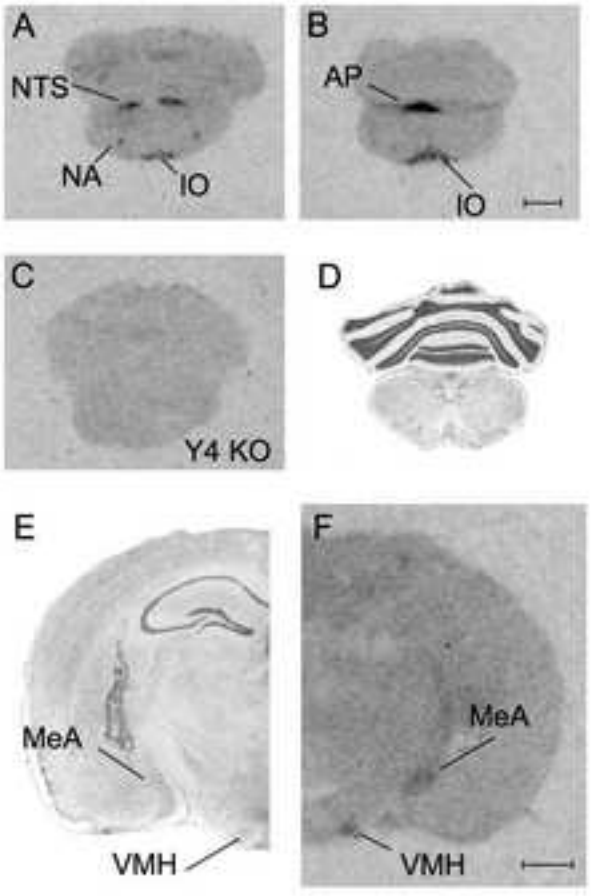

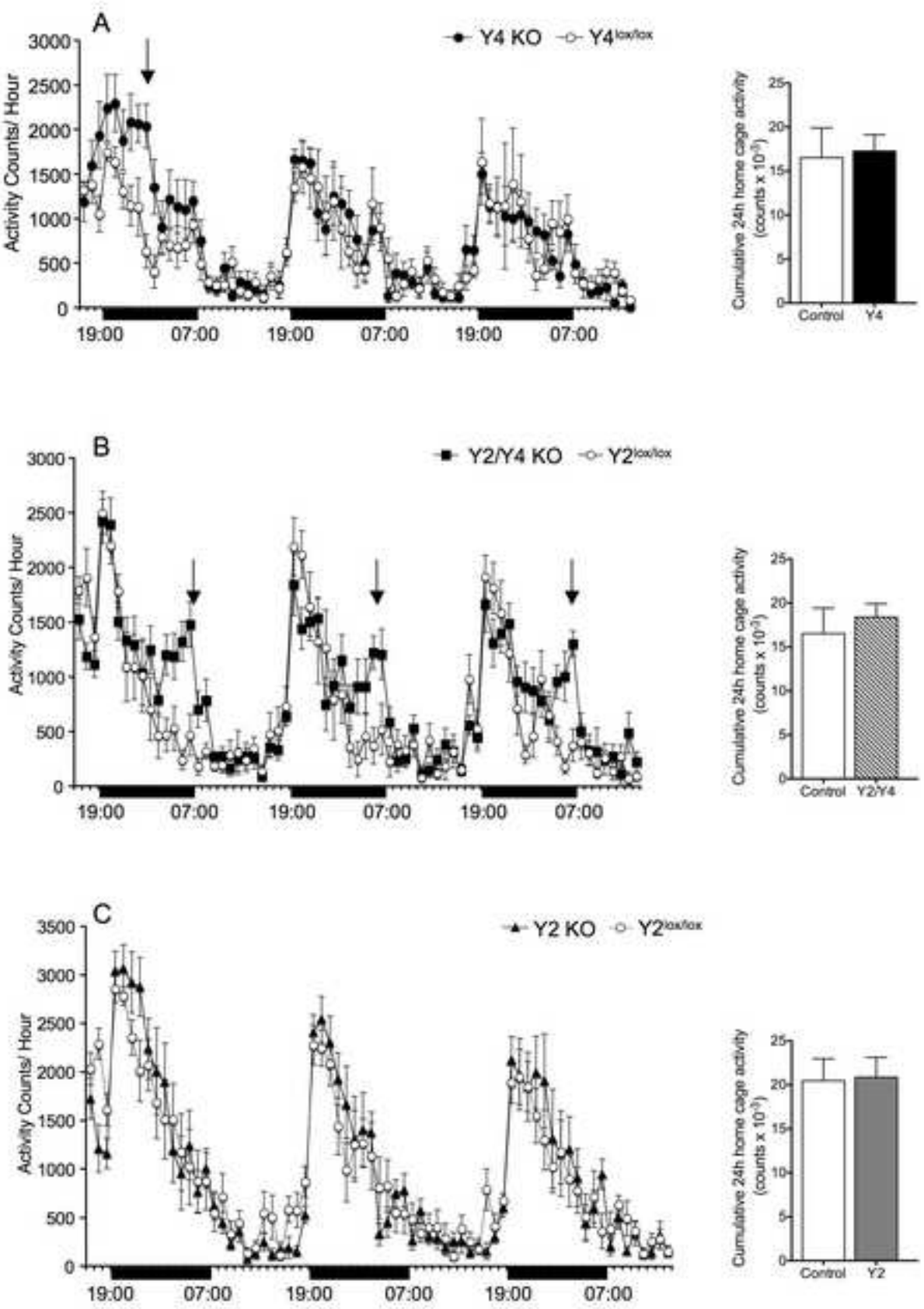

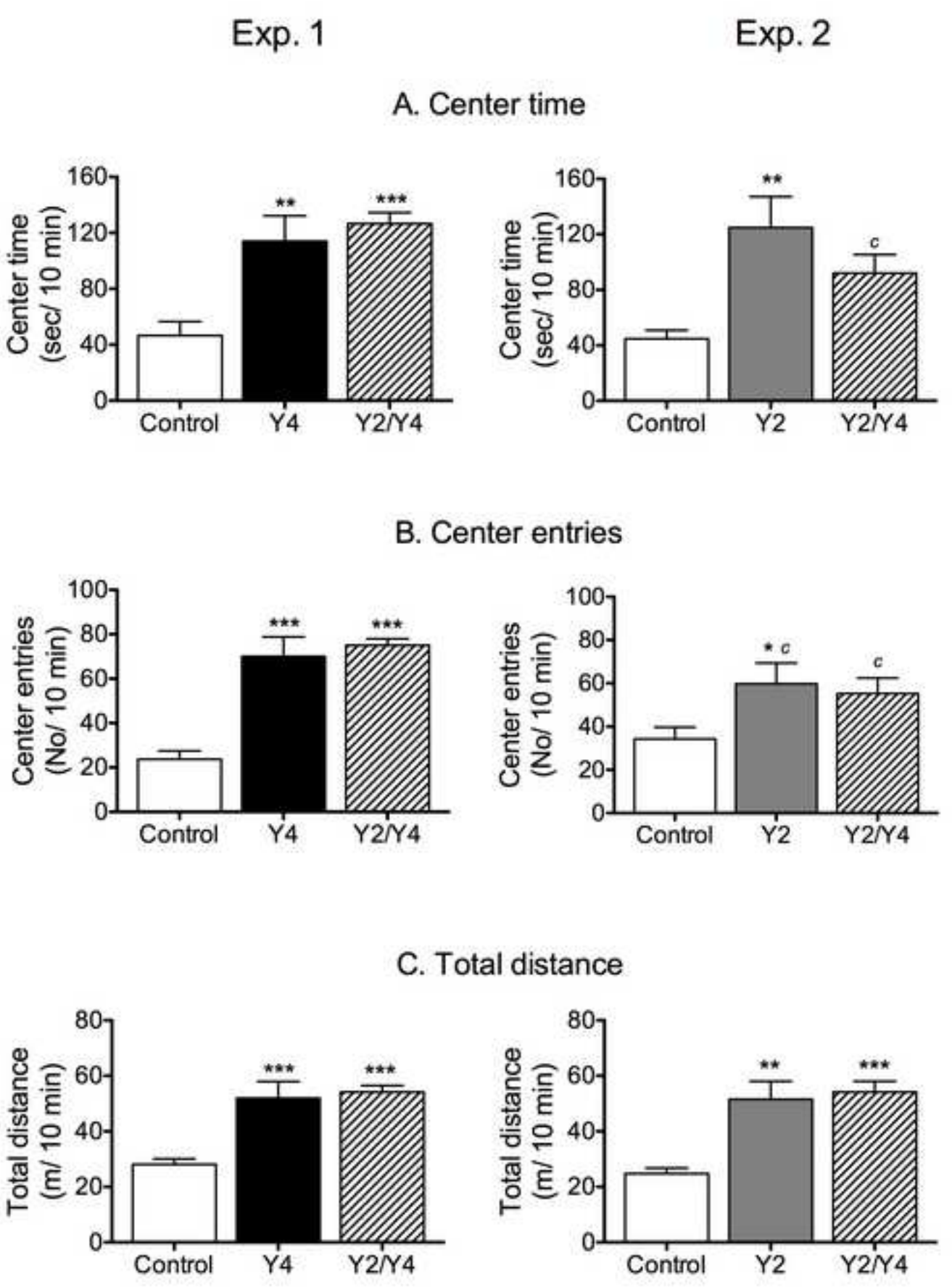

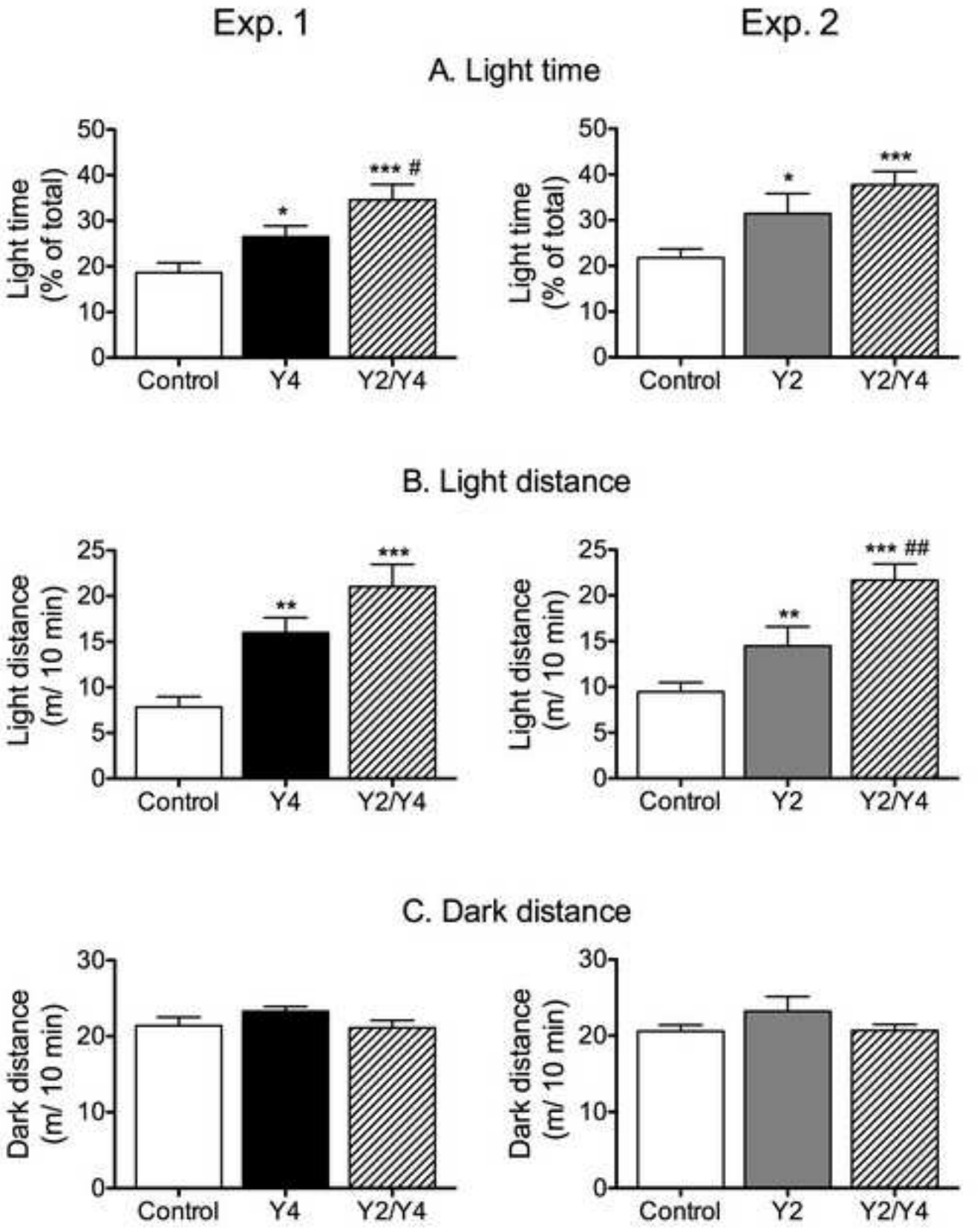

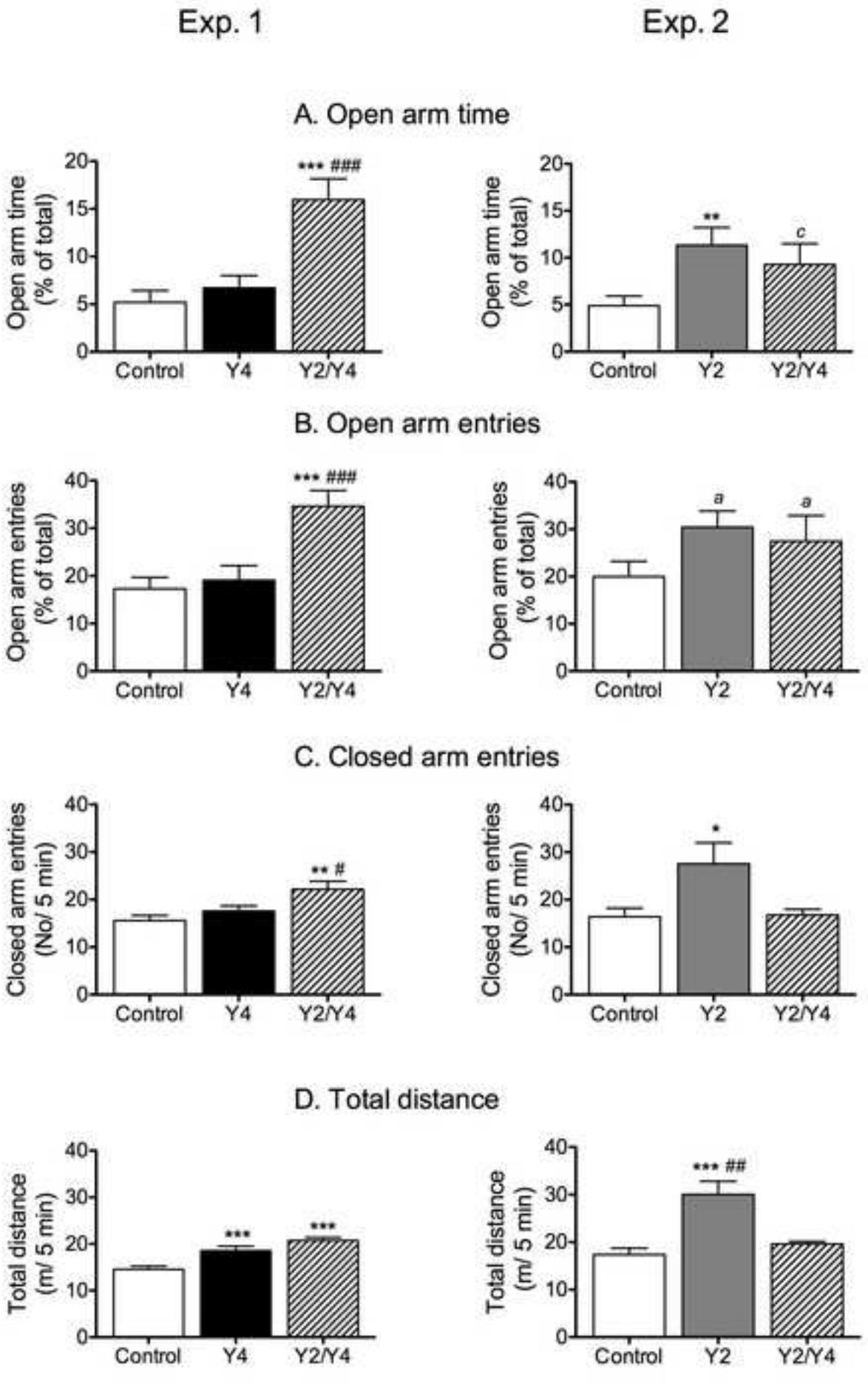

Figure 6

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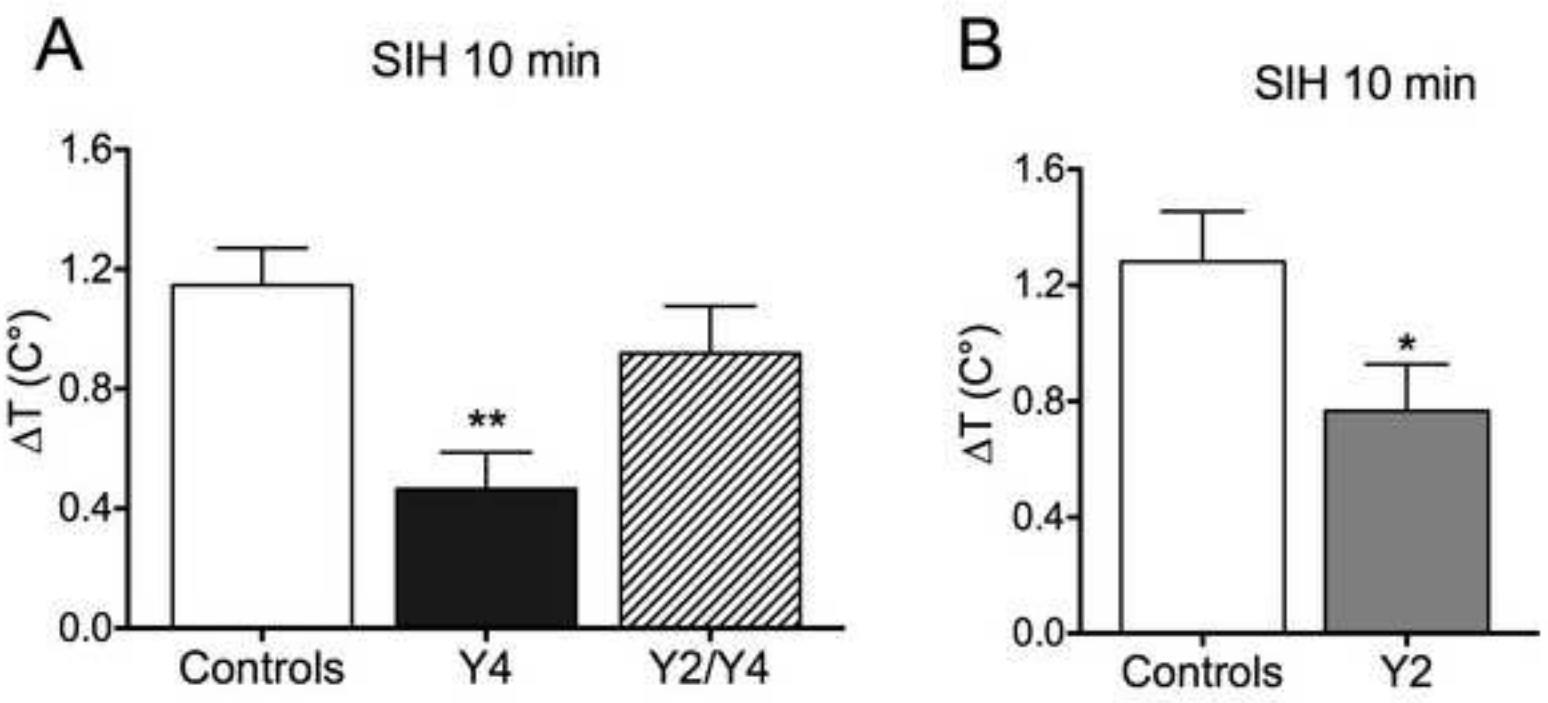

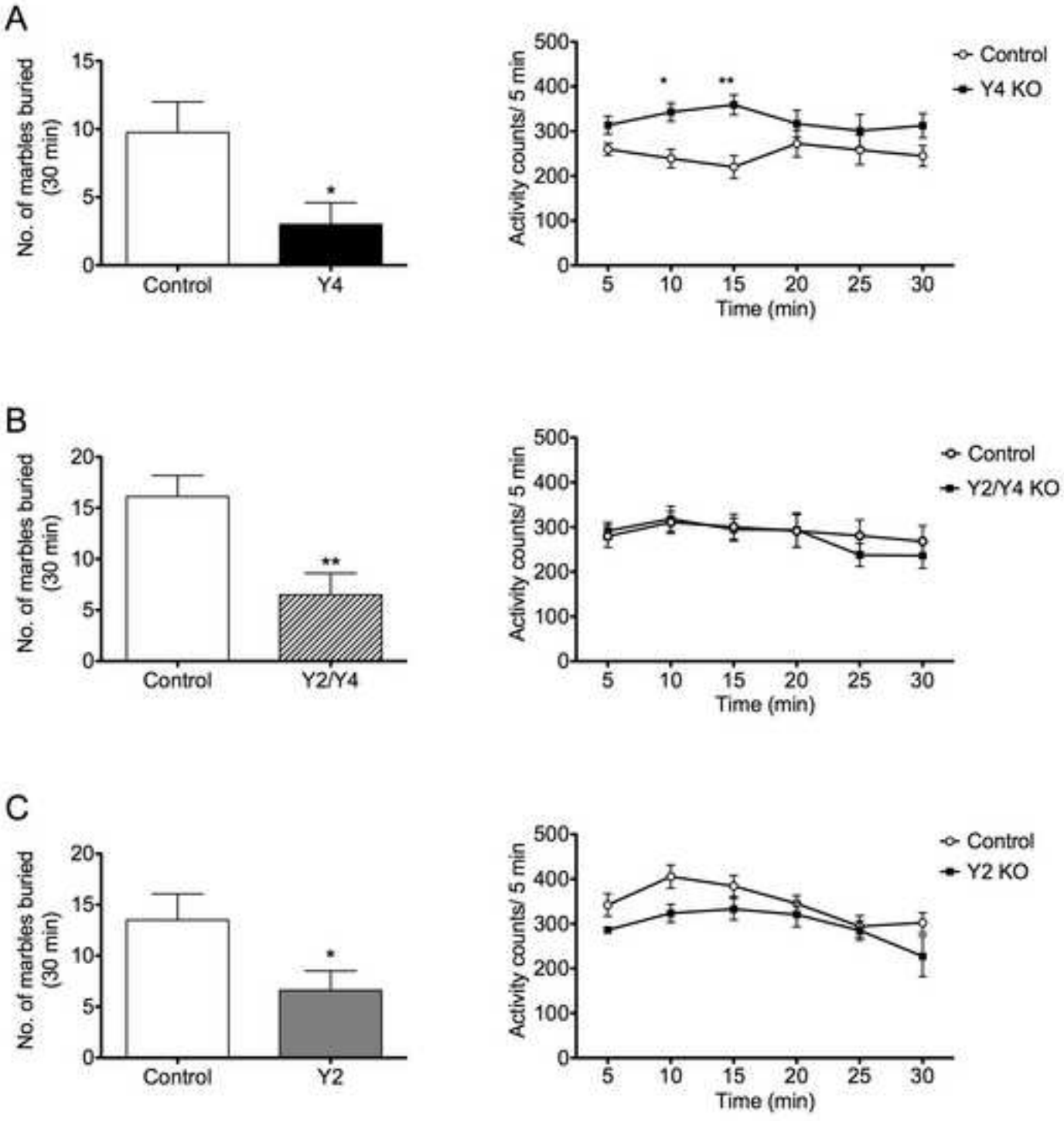

Figure 8

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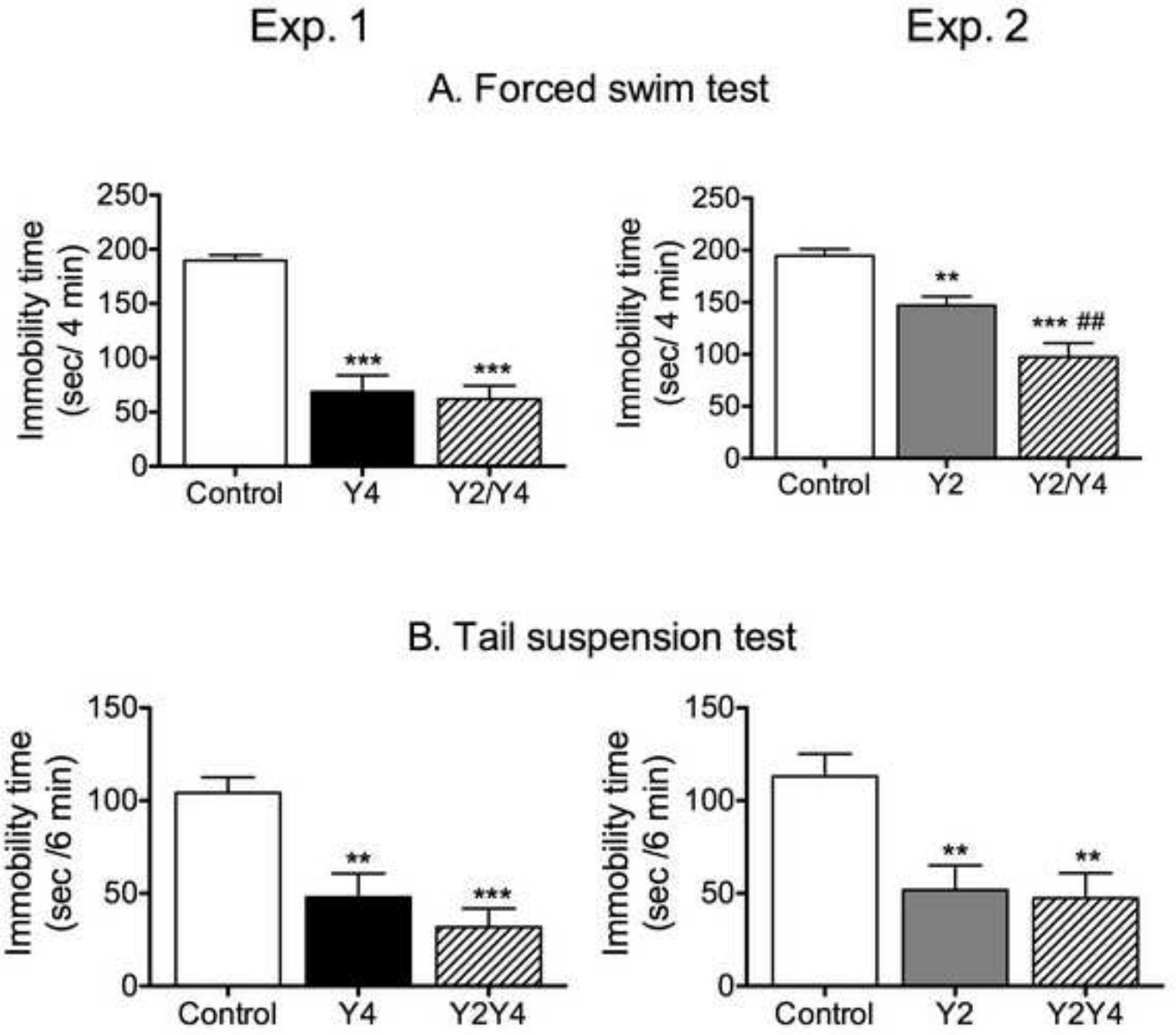

Figure 9

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