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NPY-Y1 coexpressed with NPY-Y5 receptors modulate anxiety but not mild social stress response in mice

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ABSTRACT

The Y1 and Y5 receptors for neuropeptide Y have overlapping functions in regulating anxiety. We previously demonstrated that conditional removal of the Y1 receptor in the Y5 receptor expressing neurons in juvenile Npy1r$^{Y5R-/-}$ mice leads to higher anxiety but no changes in hypothalamus-pituitary-adrenocortical axis activity, under basal conditions or after acute restraint stress. In the present study, we used the same conditional system to analyze the specific contribution of limbic neurons coexpressing Y1 and Y5 receptors on the emotional and neuroendocrine responses to social chronic stress, using different housing conditions (isolation versus group-housing) as a model.

We demonstrated that control Npy1r^{2lox} male mice housed in groups show increased anxiety and hypothalamus-pituitary-adrenocortical axis activity compared with Npy1r^{2lox} mice isolated for six weeks immediately after weaning. Conversely, Npy1r$^{Y5R-/-}$ conditional mutants display an anxious-like behavior but no changes in hypothalamus-pituitary-adrenocortical axis activity as compared with their control littermates, independently of housing conditions. These results suggest that group housing constitutes a mild social stress for our B6129S mouse strain and they confirm that the conditional inactivation of Y1 receptors specifically in Y5 receptor containing neurons increases stress-related anxiety without affecting endocrine stress responses.

INTRODUCTION

Neuropeptide Y (NPY) is a widely distributed peptide in the CNS, where it regulates anxiety and stress responses (Eva *et al.*, 2006). Pharmacologic and genetic studies suggest that NPY induces anxiolytic effects (Dumont *et al.*, 2009, Eva *et al.*, 2006, Kormos & Gaszner, 2013) and counteracts corticotropin-releasing hormone (CRH)-mediated stress responses (Russo *et al.*, 2012, Sajdyk *et al.*, 2006) via the activation of Y1 receptor (Y1R) in amygdala, hippocampus, and locus coeruleus (Bertocchi *et al.*, 2011, Broqua *et al.*, 1995, Heilig *et al.*, 1993, Lin *et al.*, 2010, Sajdyk *et al.*, 2008). In the rodent brain, the Y1R colocalizes with Y5 receptor (Y5R) in the hippocampus and basolateral amygdala (BLA) (Oberto *et al.*, 2007, Wolak *et al.*, 2003) where they have overlapping functions in regulating anxiety (Sorensen *et al.*, 2004). We previously showed that conditional inactivation of *Npy1r* restricted to Y5R-containing neurons in adult Npy1r$^{Y5R-/-}$ mice increased anxiety without affecting basal or acute stress-activated hypothalamus-pituitary adrenocortical (HPA) axis activity (Longo *et al.*, 2014). These data suggest that NPY, acting on Y1R, might activate distinct neuronal circuits to regulate anxiety-related behaviors and stress responses.

Isolation rearing in rats constitutes a stress in terms of social deprivation and adversely impacts cognitive, emotional and social functions (Lyons *et al.*, 1999, Ruis *et al.*, 1999, Sapolsky *et al.*, 1997, Serra *et al.*, 2008). Social isolation in rats induces alterations in the structure and function of $GABA_A$ receptors and decreases the cerebrocortical concentrations of 3α,5α-TH PROG (Serra et al., 2000), a neuroactive steroid derived from progesterone that binds, with high affinity, $GABA_A$ receptors (Lambert *et al.*, 1995, Majewska *et al.*, 1986, Paul & Purdy, 1992), thereby eliciting anxiolytic, anticonvulsant and hypnotic–anesthetic effects in vivo (Belelli *et al.*, 1989, Bitran *et al.*, 1993, Concas *et al.*, 1996, Kokate *et al.*, 1999).

Isolation in adult male mice may not be markedly stressful because of their territoriality (Brain, 1975, Palanza *et al.*, 2001). In fact, isolation rearing in male mice increases locomotor activity in response to novelty and decreases anxiety-like behavior (Abramov *et al.*, 2004, Bartolomucci *et al.*,

2003, Guo *et al.*, 2004, Hilakivi *et al.*, 1989, Voikar *et al.*, 2005), suggesting an improved ability to cope with novel situations.

To further explore the specific contribution to stress responses of Y1R coexpressed with Y5R in limbic neurons, we investigated the effect of chronic stressful social context, induced by different housing conditions (isolation versus group-housing with siblings), on the emotional and neuroendocrine functions of Npy1r$^{Y5R-/-}$ conditional mutants and Npy1r^{2lox} control mice. We demonstrate that group-housed Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice showed increased anxiety and HPA axis activity when compared with Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice isolated for six weeks immediately after weaning, respectively. Conversely, Npy1r$^{Y5R-/-}$ mice display an anxious-like behavior but no changes in HPA axis activity as compared with their control littermates, independently of housing conditions. This suggests that, in this B6129S mouse strain, group housing is a chronic stressful social context and the anxiolytic Y1R specifically expressed in Y5R containing neurons is not involved in stress responses.

MATERIALS AND METHODS

Animals and housing

Mice were housed in a temperature (22 ± 1°C) and humidity-controlled (50 ± 10%) room on a 12-hour light/dark cycle (8:00AM–8:00PM) and had ad libitum access to food and water. All experiments were conducted in accordance with the European Community Council Directive of 24 November 1986 86/609/EEC and 6106/10/EU and approved by the University of Turin Ethical Committee for animal research and by the Italian Ministry of Health (License No. 180/2006-B). The generation of Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ B6129S (129/SvJ, C57BL/6N derived strain) mice was achieved as previously described (Longo *et al.*, 2014).

Briefly, a targeting vector for homologous recombination in embryonic stem (ES) cells was designed to introduce loxP sites around exons 2–3, which code for the entire region of Npy1r, and a frt-neo-frt cassette ≈450 bp excised from p146 (kind gift of William Wisden) upstream the ATG of

Npy1r exon 2. The linearized targeting vector was electroporated into 5×107 R1 ES cells (129Sv x 129sv-CP) (Nagy et al., 1993) to create a conditional Npy1r allele ($Npy1r^{+/loxP\text{-}neo}$), containing two loxP sites and a frt flanked neomycin selection cassette. R1-ES cells of a correct targeted clone for the recombination ($Npy1r^{+/loxP\text{-}neo}$) were then expanded for injection into C57BL/6N blastocysts that were implanted into pseudopregnant ICR female recipients. We obtained 11 chimeric founders and three chimeric males transmitted the R1-ES cells and produced offspring with agouti coat color. PCR and Southern blot analysis of tail DNA revealed that 50% of the pups contained the gene targeted Npy1r allele. Subsequently, the *neo gene* was removed by flp-mediated recombination *in vivo*, by crossing $Npy1r^{+/loxP\text{-}neo}$ offsprings with flp expressing C57BL/6N FLP deleter mice (Dymecki, 1996). To obtain mice with *Npy1r* gene inactivation restricted to Y5R expressing neurons ($Npy1r^{Y5R\text{-}/\text{-}}$) three different mouse lines [$Npy1r^{2lox}$, $Tg^{Y5RitTA/Y1RVenus}$ mice (Oberto *et al.*, 2007), and Tg^{LC1} (Schonig *et al.*, 2002)] underwent specific breeding protocol: we generated (i) a $Npy1r^{2lox/TgY5RitTA/Y1RVenus}$ mouse line, expressing the tTA in a cell type-specific manner under the control of the *Npy5r* promoter and (ii) a $Npy1r^{2lox/TgLC1}$ line, encoding the tTA-responsive Cre transgene Tg^{LC1}. Region-specific inactivation of *Npy1r* was achieved by crossing $Npy1r^{2lox/TgY5RitTA/Y1RVenus}$ with $Npy1r^{2lox/Tg\ LC1}$ mice. Doxycycline treatment of mothers (50 mg/L in drinking water, 1% sucrose) from conception to birth keeps the Cre transgene inactive in embryos and synchronizes the postnatal Cre activation. In $Npy1r^{2lox/Y5RitTA/\ Y1RVenus/LC1}$ mice ($Npy1r^{Y5R\text{-}/\text{-}}$ mice) Cre expression was fully activated at approximately P40. $Npy1r^{2lox}$ ($Npy1r^{2lox/TgY5RitTA/Y1RVenus}$, $Npy1r^{2lox}$, $Npy1r^{2lox/Tg\ LC1}$) and $Npy1r^{2lox/TgY5RitTA/\ Y1RVenus\ /LC1}$ ($Npy1r^{Y5R\text{-}/\text{-}}$) were maintained in a randomly segregating population derived from more than 300 different breeding pairs and each litter provided the same number of experimental animals to both $Npy1r^{2lox}$ and $Npy1r^{Y5R\text{-}/\text{-}}$ groups. $Npy1r^{2lox}$ control mice ($Npy1r^{2lox/TgY5RitTA/Y1RVenus}$, $Npy1r^{2lox}$, $Npy1r^{2lox/Tg\ LC1}$) didn't show any recombination of the floxed *Npy1r* allele and were used as control littermates in all experiments (Crusio *et al.*, 2009).

For the maintenance of the gene targeted and the transgenic lines animals were backcrossed to C57BL/6N mice. The coat color of the investigated animals was still mixed 'mainly black'

indicating that the genetic background of the investigated population is very heterogeneous, which suggests that the observed phenotype is independent from the genetic background (Silva et al., 1997).

Immediately after weaning (4 weeks), male mice were assigned into four different groups: Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ single-housed and Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ group-housed with siblings (3-4 Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ brothers per cage). After six weeks, mice were tested for anxiety-like behavior (69 mice) and, the day after, weighed and sacrificed for in situ hybridization or perfused for immunohistochemical analysis. For 3α, 5α-TH PROG quantification, 27 mice were immediately sacrificed after 6 weeks of different housing conditions. None of the animals were excluded from the analysis.

In situ hybridization for *Npy1r* mRNA

In situ hybridization was performed on coronal brain sections (14 µm thick). Four different 40-/45-mer oligonucleotide S35-labeled probes were simultaneously used to increase the signal and the reaction was carried out as previously described (Longo *et al.*, 2014), according to the protocol reported by Wisden and Morris (Wisden & Morris, 2002). The area of interest of clearly distinguishable nuclei was defined following the boundaries of the labeled regions [CA1 pyramidal cell layer (CA1), dentate gyrus granule cell layer (DG)], while three to four spots were used for each slice in poorly contrasted regions [basolateral amygdala (BLA) and CA3 pyramidal cell layer (CA3)]. Optical densities were measured and averaged using a Rodbard calibration to convert gray levels in Optical Density (OD) unit, following the instruction in ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014). Background was measured by averaging three to five spots [optical density (OD) unit] in the surrounding blank region of the autoradiogram then subtracted from the correspondent nucleus value. Brain nuclei coordinates relative to bregma line were: BLA about -1.46 to -1.70 mm; DG, granular layer -1.46 to -1.70 mm; CA1 and CA3 (pyramidal layer) -1.58 to -1.94 mm.

Immunohistochemistry

Mice were given an anesthetic overdose of Zoletil (25 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS). Immunostainings of corticotropin releasing hormone (CRH) and glucocorticoid receptor (GR) were carried out on free-floating brain coronal sections (25 μm), using the ABC Vectastain kit (Vector Laboratories, Burlingame, CA, USA). For CRH, after endogenous peroxidase blocking in 2% H_2O_2 in 0.1 M PBS for 30 min, sections were blocked with 10% normal goat serum (NGS) and incubated for 48 h with primary rabbit CRH antibody (1:4000; Peninsula Laboratories Bachem, UK). After washing in PBS 0.3% Triton sections were incubated for 1 h with a secondary biotinylated goat anti-rabbit antibody (1:300; Vector Laboratories) followed by the ABC reagent. Peroxidase enzyme activity was visualized by 0.05% diaminobenzidine tetrahydrochloride (DAB; SigmaAldrich, Milan Italy). For each animal three to five sections of central amygdala (CeA) (-1.46 mm relative to bregma) and three to four sections of medial parvocellular division of the paraventricular nuclei (PVNmp) (-0.82 mm relative to bregma) were analyzed. For GR, after endogenous peroxidase blocking in 0.5% H_2O_2 in 0.1 M PBS for 15 min, sections were blocked with 10% NGS in DayI buffer (PBS containing 0.3% Triton X-100 and 1% bovine serum albumin) for 1 h and incubated overnight at room temperature with primary GR antibody (Santa Cruz Biotechnology, Heidelberg, Germany) at 1:1000 dilution in DayI solution. After washing in DayII buffer (1:3 dilution of DayI in 0.1M PBS), sections were incubated for 1 h with a secondary biotinylated goat anti-rabbit antibody (1:300; Vector Laboratories) followed by the ABC reagent. Peroxidase was reacted with 0.05% DAB and 0.003% H_2O_2, mounted on slides and air-dried overnight. For each animal, five to seven sections of CA1 were analyzed. ImageJ software (NIH) was used to measure CRH and GR immunoreactivities through a threshold method and the number of positive pixels and the extension of area of interest were used to determine the fractional area covered by the specific signal.

Immunofluorescence

Immunostaining of Y1R was carried on free-floating vibratome-cut brain coronal sections (50 μm) as previously described (Longo *et al.*, 2014). Primary Y1R antibody was kindly provided by Janice

Urban and was used at 1:2000 dilution. Briefly, after the incubation with the biotinylated secondary antibody, floating sections were subjected to tyramide amplification (TSA Fluorescence System, Perkin Elmer). Sections were then mounted and coverslipped with Mowiol and pictures of brain regions were taken with a confocal microscope (Nikon D-Eclipse C1, Nikon).

Behavioral studies

At the end of the 6 weeks of isolation or group-housing, elevated plus maze (EPM) and open field (OF) were performed close to shifting phase timings (from 8 to 10 AM for the OF or 5–7 PM for EPM), when mice are more active. In the groups of mice that were analyzed by both of the tests, EPM and OF were performed the same day. On test day, mice were transported to the testing room and left undisturbed for 1 h before testing. The experiments were performed under dim white light conditions (2 lux). For the EPM, mouse was placed in the center of the plus maze [comprising two open arms (30 cm x 5 cm x 0.20 cm, light intensity 5.3 lux) and two closed arms (30 cm x 5 cm x 15 cm walls, light intensity 3.5 lux)], facing an open arm and it was allowed to explore the maze for 5 min. The cumulative time spent in and the number of entries into open and closed arms were recorded. For the OF, animal was placed in the corner of the arena (50 x 50 x 50 cm) with a light intensity of 1.4 lux, for 5 min. Total distance traveled, time spent and number of entries in the central area (25 x 25) were recorded. At the end of each trial, the apparatus was accurately cleaned up with ethanol 2% and water. Data were analyzed using Ethovision XT9 video track system (Noldus Information Technology, Wageningen, The Netherlands).

Serum collection and analysis

A group of mice that were analyzed by both the EPM and the OF were given an anesthetic overdose of Zoletil (25 mg/kg, i.p.) 24-h after the beginning of behavioral tests. Blood samples were collected from the atrium, centrifuged, and serum was frozen at −20°C until analyzed. Serum was assayed for corticosterone levels by using commercially available kits (MP Biomedicals; RIA).

3α, 5α TH-PROG extraction and assay

Steroids present in cerebral cortical homogenates were extracted three times with ethyl acetate, and the combined organic phases were dried under vacuum. The resulting residue was dissolved in 5mL of *n*-hexane and applied to a SepPak silica cartridge (Waters, Milford, MA, USA), and components were eluted with *n*-hexane and 2-propanol (7 : 3, v/v). Steroids were separated and further purified by high-performance liquid chromatography (HPLC) on a 5-µm Lichrosorb-diol column (250 by 4 mm; Phenomenex, Torrance, CA, USA) with a discontinuous gradient of 2-propanol (0–30%, v/v) in *n*-hexane. The recovery (70–80%) of 3α, 5α TH-PROG through the extraction and purification procedures was monitored by adding trace amounts of tritiated 3α, 5α TH-PROG (6000–8000 cpm; 20–80 Ci/mmol) to the cortical homogenate. 3α, 5α TH-PROG was quantified by radioimmunoassay as previously described (Serra *et al.*, 2000) with a specific antibody to 3α, 5α TH-PROG (Purdy *et al.*, 1990). Protein concentration was measured by the method of Lowry (1951) with bovine serum albumin as standard.

Statistical analysis

All the quantitative results were analyzed by two-way analysis of variance, followed by Newman-Keuls test, for multiple comparisons. Data were analyzed using the STATISTICA software (StatSoft, Inc). All data are expressed as mean ± SEM, and the level of statistical significance was set at $p<0.05$.

RESULTS

Effect of housing conditions on *Npy1r* expression

The effect of different housing conditions and genotype on *Npy1r* gene expression were first analyzed by semiquantitative *in situ* hybridization. No differences of *Npy1r* mRNA expression were observed in the cerebral cortex, in the hippocampal CA1 and CA3 pyramidal cell layers, in the dentate gyrus (DG) granule cell layer, and in the BLA of group-housed compared to isolated $Npy1r^{2lox}$ control mice. The conditional inactivation of *Npy1r* led to a significant reduction of *Npy1r* mRNA in the hippocampus and BLA of $Npy1r^{Y5R-/-}$ mice, compared with $Npy1r^{2lox}$ mice, as

previously demonstrated (Longo *et al.*, 2014) (Fig. 1a and 1b). Two-way ANOVA for genotype and housing conditions indicated a significant effect of genotype in the CA1 [F(1,9) = 24.29; P= 0.0008] and CA3 [(F(1,9) = 17.26; P= 0.0025)] pyramidal cell layers, in the DG granule cell layer [F(1,9) = 43.92; P= 0.0001], and in the BLA [F(1,9) = 6.73; P = 0.0289]. Accordingly, the immunofluorescence staining of coronal brain sections using an anti-Y1R antibody confirmed that housing conditions did not influence the expression of Y1R in the hippocampus and the amygdala of isolated and group-housed Npy1r^{2lox} mice, whereas conditional inactivation of *Npy1r* led to a reduction of Y1R expression in the CA1 and CA3, in the DG, and in the BLA of Npy1r$^{Y5R-/-}$ mice, compared with Npy1r^{2lox} mice (Fig. 1c).

Effect of housing conditions on anxiety behavior of Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice

Group-housed Npy1r^{2lox} control mice showed increased anxiety-like behavior in the EPM (Fig. 2a) and in the OF (Fig. 2b) compared with isolated Npy1r^{2lox} control mice. Npy1r$^{Y5R-/-}$ mice show increased anxiety-related behavior in both of the tests compared with Npy1r^{2lox} siblings, independently of the housing conditions (Fig. 2 a and b). In the EPM test, two-way ANOVA for housing conditions and genotype revealed a significant effect of housing conditions and genotype for percent of entries in open arms [F(1,65) = 33.32, P=0.0001 and F(1,65) = 7.57, P = 0.0077, respectively], and of housing conditions, genotype, and housing conditions and genotype interaction for percent of time in open arms [F(1,65) = 24.88, P = 0.0000; F(1,65) = 9.61, P = 0.0029 and F(1,65) = 7.16, P = 0.0094, respectively]. Consistently, group-housed mice showed a significant decrease of total entries in the arms, compared with isolated mice (isolated Npy1r^{2lox}: 36±3.1; isolated Npy1r$^{Y5R-/-}$: 38±2.7; group-housed Npy1r^{2lox}: 31±2.3; group-housed Npy1r$^{Y5R-/-}$: 31±2.3) suggestive of a lower exploratory behavior [two-way ANOVA for housing conditions: F(1,65) = 4.691, P = 0.034]. The significant effect of housing conditions and genotype interaction for percent of time in open arms seems to be mainly due to the large differences in the absolute values between isolated and group-housed mice that are more anxious than isolated mice. In OF test, there was a significant effect of housing conditions and genotype for distance travelled in the center [F(1,28) =

4.33, P= 0.047 and F (1,28) = 5.56, P=0.026, respectively]. A similar effect, although not significant, of housing conditions and genotype was observed in the number of total entrance in the center of the OF (isolated Npy1r^{2lox}: 16.8±4.1; isolated Npy1r$^{Y5R-/-}$: 10.8±2.7; group-housed Npy1r^{2lox}: 12.8±2.6; group-housed Npy1r$^{Y5R-/-}$: 8.8±2.9) and in the % of time spent in the center of the OF (isolated Npy1r^{2lox}: 7.7±0.7; isolated Npy1r$^{Y5R-/-}$: 5.6±1.8; group-housed Npy1r^{2lox}: 6.5±0.9; group-housed Npy1r$^{Y5R-/-}$: 3.9±1.1). Housing conditions and genotype failed to affect locomotor activity of Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice, as evidenced by total distance moved in the OF (Fig. 2b).

Effect of housing conditions on physiological functions of Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice

Independently of genotype, group-housed mice displayed lower density of CRH immunoreactive fibers in the central amygdala (CeA) and of glucocorticoid receptor (GR) immunoreactive neurons in the CA1, and higher density of CRH cell bodies in the medial parvocellular division of the paraventricular nuclei (PVNmp) and of corticosterone serum levels, compared with isolated mice, which suggests increased central drive of the HPA-axis activity (Bertocchi *et al*., 2011, Dimitrov *et al.*, 2007, Noguchi *et al.*, 2010) (Fig. 3 and Fig. 4). Mice raised in group also showed higher cerebrocortical concentrations of 3α, 5α TH-PROG than isolated mice (Fig. 3).

Two way ANOVA revealed a significant effect of housing conditions for CRH immunoreactivity (IR) in the CeA [F(1,21)=6.364, P=0.020], CRH-IR in the PVNmp [F(1,27)=21.2, P=<0.001], GR-IR [F(1,15)=13.815, P=0.0021], corticosterone [F(1,19)= 8.51, P=0.0089] and 3α, 5α TH-PROG [F(1,23)= 26.25, P=0.0000]. No differences in CRH-IR and GR-IR, corticosterone and 3α, 5α TH-PROG, were observed between Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice, independently of housing conditions. No significant differences in body weight were observed among experimental groups ([g], isolated Npy1r^{2lox} = 32.4 ± 1.3; isolated Npy1r$^{Y5R-/-}$ = 29.9 ± 0.8; group-housed Npy1r^{2lox} = 29.4 ± 1.5; group-housed Npy1r$^{Y5R-/-}$ = 28.5 ± 0.9).

DISCUSSION

Animal models that involve a social context, such as housing conditions, are more appropriate than conventional animal models of stress, including restraint, to mimic a stressful situation that an animal can meet in its real life events and for which behavioral and neuroendocrine responses had been shaped by evolutionary processes (Palanza *et al.*, 2001). A number of studies have clearly demonstrated that group housed male mice may show behavioral (e.g. Barnard *et al.*, 1991, Kaliste-Korhonen & Eskola, 2000), endocrine (e.g. Brain & Benton, 1983) and immune (e.g. Bohus & Koolhaas, 1991) indices of stress as a consequence of aggressive interactions between cage-mates.

In accordance with previous studies (Wersinger *et al.*, 2002), we observed a high level of aggression of Npy1r^{2lox} mice, compared to typical levels observed in the C57BL/6N strain, possibly due their mixed genetic background (P. Palanza, personal communication).

Here we showed that group-housing of our mouse strain increases anxiety-like behavior in the EPM and in the OF and activates HPA axis, as shown by the increase of corticosterone serum levels and CRH-IR in PVNmp and the decrease of CRH-IR in CeA and GR-IR in the CA1 (Noguchi *et al.*, 2010). These findings are consistent with previous studies showing that individually housed male mice of different strains showed decreased anxiety-like behavior in different experimental paradigms (Abramov *et al.*, 2004, Bartolomucci *et al.*, 2003, Guo *et al.*, 2004, Hilakivi *et al.*, 1989, Palanza *et al.*, 2001, Voikar *et al.*, 2005), and lower corticosterone plasma levels compared with their group-housed counterpart males (Mucignat-Caretta *et al.*, 2014). However, contrasting reports on the possible stressfulness of isolation in mice exist, and other studies found opposite or no effects of group housing on emotional behavior and endocrine system (Arndt *et al.*, 2009, Koike *et al.*, 2009, Liu *et al.*, 2013, Zhang *et al.*, 2012). Differences among studies in the species, the laboratory strain, the duration and timing of isolation and the procedural variables in testing anxious behavior may explain these discrepancies. Thus, we cannot rule out that the lack of backcrossing of our B6129S mouse strain onto C57BL/6N strain of mice could impact on the results (Crusio *et al.*, 2009).

Several lines of evidence suggest that, in rodents, NPY participates in the regulation of anxious

responses (Dumont *et al.*, 2009, Dumont & Quirion, 2014, Eva *et al.*, 2006, Kormos & Gaszner, 2013) and has marked anti-stress effects (Russo *et al.*, 2012, Sajdyk *et al.*, 2006) via the activation of Y1R in amygdala, hippocampus, and locus coeruleus (Bertocchi *et al.*, 2011, Broqua *et al.*, 1995, Heilig *et al.*, 1993, Lin *et al.*, 2010, Sajdyk *et al.*, 2008). Genetic and pharmacologic experiments suggested that in addition to the Y1R, the Y5R subtype in the BLA plays a significant role in anxiety-related behaviors (Domschke *et al.*, 2008, Roseboom *et al.*, 2014, Sajdyk *et al.*, 2002, Sorensen *et al.*, 2004).

We have previously shown that the conditional inactivation of *Npy1r* selectively in neurons co-expressing Y5R increases anxiety but is not associated with the activation of HPA axis activity (Longo *et al.*, 2014). In the present study we further demonstrated that Npy1r$^{Y5R-/-}$ conditional mutants display a strong anxious phenotype in the EPM and OF, independently of housing conditions, but show identical levels of CRH in the CeA and PVNmp, of GR in the CA1 and of corticosterone serum levels, compared to their control littermates. These data confirm that Y1Rs expressed in Y5R containing neurons in the limbic system play a major role in the control of anxious behavior but fails to affect stress-related markers. This suggest that NPY, acting on Y1R, might activate distinct neuronal circuits to regulate anxiety- and stress-related responses. On the other hand, given that Y1R and Y5R colocalization may result in receptor heterodimerization and in enhanced or altered function of several physiologic systems (Mashiko *et al.*, 2009), the anxious phenotype of Npy1r$^{Y5R\ -/-}$ mice might be due to both the conditional inactivation of *Npy1r* and the Y5R altered expression/function.

Experimental evidence suggested a functional interaction between GABA and NPY that may be important in the regulation of anxiety-like behavior (Dimitrov *et al.*, 2007, Sajdyk *et al.*, 2006).

The BLA is an important component of neuronal circuitry controlling anxiety-related behaviors where both the Y1R and the Y5R appear to be required for the anxiolytic response to NPY. Previous studies suggested that, in the rodent limbic system, the Y1R and Y5R are colocalized in the BLA (Oberto *et al.*, 2007, Wolak *et al.*, 2003) and are coexpressed in 50% of GABAergic

interneurons (Longo *et al.*, 2014), which have strong synaptic control over principal neurons - the pyramidal cells (Muller *et al.*, 2006, Rainnie *et al.*, 2006). Therefore, the inactivation of *Npy1r* in neurons co-expressing Y5R could have profound effects on the synchronous activity of the BLA and may explain the phenotype of Npy1r$^{Y5R-/-}$mice. The amygdala was identified as a key structure for mediating the anxiolytic effects of 3α,5α-TH PROG, as its direct infusion into the amygdala was followed by a significant increase in the number of entries and the time spent in the open arms of the EPM (Akwa et al., 1999) and its anxiolytic-like effects vary as a function of intracerebral microinfusion site (Engin & Treit, 2007). We have previously shown that a sustained increase in the brain concentrations of neuroactive steroids, induced by pharmacological treatment or by physiological conditions, results in the upregulation of *Npy1r* gene expression in the amygdaloid complex, suggesting that these compounds might play an important functional role in regulating the modulatory effect of NPY on emotional behavior (Eva *et al.*, 2008, Ferrara *et al.*, 2001, Oberto *et al.*, 2002).

Conversely, adaptation of GABAergic transmission to chronic stress, apparently, does not involve Y1R-mediated transmission in the amygdala, since exposure to repeated restraint stress, which induced repetitive and transient increases in the concentrations of 3α,5α-TH PROG in the cerebral cortex, failed to affect *Npy1r* gene expression in this region (Mele *et al.*, 2004).

Accordingly, here we showed that group housing increases the concentrations of 3α,5α-TH PROG in the cerebral cortex of both Npy1r$^{Y5R-/-}$ and Npy1r^{2lox} mice, compared with their isolated counterparts.

The finding that both chronic restraint and mild social stress increase cerebrocortical concentrations of 3α,5α-TH PROG may be surprising since, consistent with its ability to facilitate GABAergic neurotransmission, this neuroactive steroid exhibits potent anxiolytic-like effects in a variety of animal models (Gasior et al., 1999). The increase in neuroactive steroid concentrations induced by chronic stress might represent a homeostatic mechanism for the restoration of $GABA_A$ receptor function and be physiologically relevant in protection against stress-induced anxiety. On the other

hand, cerebrocortical levels might not reflect dynamic release patterns into different compartments of the brain. Further investigations focused on different brain areas are needed to ascertain putative differences between socially isolated and group-housed mice. In conclusion, the current study expands on our finding on Npy1r$^{Y5R-/-}$ mice phenotype by showing that the inactivation of *Npy1r* in neurons co-expressing Y5R increases anxiety but is not involved in promoting resilience to a mild social stress, such as group housing seems to be in this B6129S mouse strain, since no changes in stress-related markers were observed between conditional mutants and their control littermates. This finding provides additional information about the NPY-related neuronal pathways involved and the specific role of Y1R coexpressed with Y5R in limbic neurons in decreasing anxious behavior and stress susceptibility, suggesting a potential strategy of novel therapeutics for a variety of anxiety- and stress-related disorders.

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FIGURE LEGENDS

Figure 1: Expression of *Npy1r* messenger RNA (mRNA) and Y1 receptor (Y1R) peptide in the brain of isolated or group-housed Npy1r^{2lox} control and Npy1r$^{Y5R-/-}$ conditional mutants.

(**a**) Representative autoradiograms of *in situ* hybridization of *Npy1r* mRNA on brain coronal sections from isolated and group-housed Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice. (**b**) Quantitative signal intensity (OD) analysis of *in situ* hybridization revealed a significant decrease of *Npy1r* mRNA expression in the DG granule cell layer, CA1 and CA3 pyramidal cell layers of hippocampus and in the BLA of Npy1r$^{Y5R-/-}$ mice compared with control Npy1r^{2lox} littermates independently of the housing conditions. Data are expressed as relative optical density and are the mean ± SEM from two independent experiments; n=3 (group-housed Npy1r$^{Y5R-/-}$ and Npy1r^{2lox}) and 4 (isolated Npy1r$^{Y5R-/-}$) mice. *P<0.05 and ** P<0.01 versus isolated or grouped-housed Npy1r^{2lox} mice, by Newman–Keuls (Scale bar = 1.5 mm). (**c**) The Y1R immunofluorescence confirmed the reduced Y1R expression in DG, CA1, and CA3 of hippocampus and in the BLA of grouped housed and isolated Npy1r$^{Y5R-/-}$ mice compared with Npy1r^{2lox} siblings (Scale bar = 50 µm). BLA, basolateral amygdala; CA1, CA1 stratum pyramidale; CA3, CA3 stratum pyramidale; DG, dentate gyrus; gr, granular cell layer; mol, molecular layer; po, polymorphic-layer; pyr, pyramidal cell layer; rad, stratum radiatum; slu, stratum lucidum.

Figure 2: Anxiety-like behavior of isolated and group-housed Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice. Two way ANOVA revealed that group housing conditions (group-housed mice versus isolated mice) and conditional inactivation of *Npy1r* in Y5R containing neurons significantly increases anxiety levels in the elevated plus maze and open field tests (see Results section). Group-housed Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice showed significantly lower frequency of entries and percent of time spent in the open arms of the elevated plus maze (**a**), and lower percent of distance travelled in the center of the open field (**b, left panel**) compared with isolated Npy1r^{2lox} mice and Npy1r$^{Y5R-/-}$ mice, respectively. In the same behavioral tests, Npy1r$^{Y5R-/-}$ mice showed an anxious phenotype compared

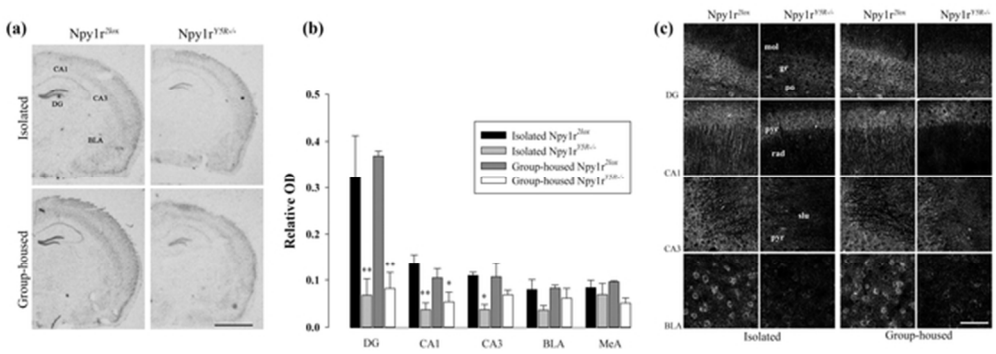
with Npy1r^{2lox} siblings, independently of housing conditions. Genotype and housing conditions failed to affect total distance travelled by Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice in the OF (**b, right panel**). EPM: data are the mean ± SEM; n=12 (isolated Npy1r^{2lox}), 15 (isolated Npy1r$^{Y5R-/-}$) and 21 (group-housed Npy1r^{2lox} and Npy1r$^{Y5R-/-}$) mice from 7 to 11 litters. **P<0.01 versus isolated Npy1r^{2lox} mice. OF: data are the mean ± SEM; n=6 (isolated Npy1r^{2lox}), 5 (isolated Npy1r$^{Y5R-/-}$), 10 (group-housed Npy1r^{2lox}) and 11 (group-housed Npy1r$^{Y5R-/-}$) mice from 4 to 5 litters.

Figure 3: Physiological functions in isolated and group-housed Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice. Two way ANOVA revealed that group housing conditions (group-housed mice versus isolated mice) significantly decreases glucocorticoid receptor immunoreactivity (GR-IR) in the CA1 and corticotropin releasing hormone immunoreactive (CRH-IR) fibers in the central amygdala (CeA) , and significantly increases CRH-IR cell bodies in the medial parvocellular division of the paraventricular nucleus (PVNmp) and corticosterone serum levels compared with isolation, which suggests increased central drive of the HPA-axis activity (see Results section). In addition, group housing significantly increases cerebrocortical concentrations of 3α,5α-TH PROG compared with isolated mice, independently of the genotype. Data are expressed as the mean ± SEM. Immunoreactivity, fractional area x 10^2: GR-IR: n=5 (group-housed Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ and isolated Npy1r^{2lox}) and 4 (isolated Npy1r$^{Y5R-/-}$) mice from 2-4 litters; CeA CRH-IR: n=7 (isolated Npy1r^{2lox} and Npy1r$^{Y5R-/-}$), 5 (group-housed Npy1r^{2lox}) and 6 (group-housed Npy1r$^{Y5R-/-}$) mice from 2-4 litters; PVNmp CRH-IR: n= 7 (isolated Npy1r^{2lox}), 8 (isolated Npy1r$^{Y5R-/-}$ and group-housed Npy1r^{2lox}) and 9 (group-housed Npy1r$^{Y5R-/-}$) mice from 2-4 litters; corticosterone serum levels (ng/ml): n= 6 (isolated and group-housed Npy1r^{2lox}), n=5 (isolated Npy1r$^{Y5R-/-}$) and n=7 (group-housed Npy1r$^{Y5R-/-}$) mice from 2-5 litters. 3α,5α-TH PROG (ng/g of tissue) n= 6 (isolated and group-housed Npy1r^{2lox}), n=8 (isolated Npy1r$^{Y5R-/-}$), n=7 (group-housed Npy1r$^{Y5R-/-}$) from 2-4 litters.

##P<0.01 versus isolated Npy1r^{2lox}; #P<0.05 versus isolated Npy1r^{2lox}; **P<0.01 versus isolated Npy1r$^{Y5R-/-}$ mice; *P<0.05 versus isolated Npy1r$^{Y5R-/-}$ mice by Newman–Keuls.

Figure 4: Representative pictures of corticotropin releasing hormone and nuclear glucocorticoid receptor immunoreactive staining of brain coronal sections from isolated and group-housed Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice.

Group housed mice showed a decrease of the density of nuclear glucocorticoid immunoreactive (GR-IR) staining in the CA1 pyramidal cell layer (**a**) and of corticotropin releasing hormone immunoreactive (CRH-IR) fibers in the central amygdala (**b**) and an increase of CRH-IR cell bodies in the medial parvocellular division of the paraventricular nuclei (PVNmp) (**c**) compared to isolated mice, independently of the genotype. (Scale bar = 100 µm).



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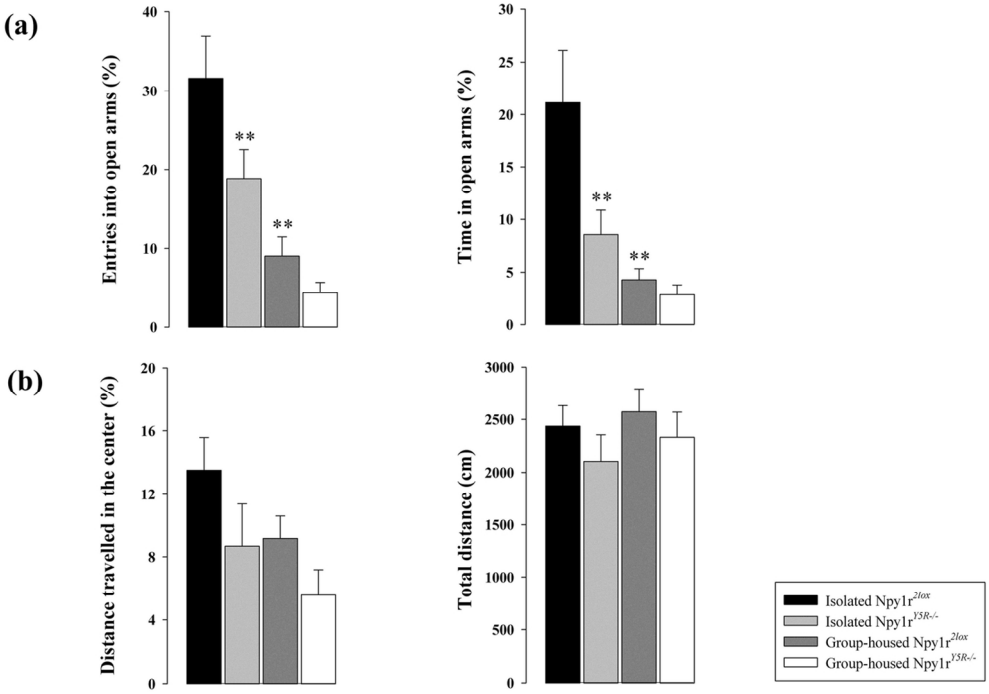
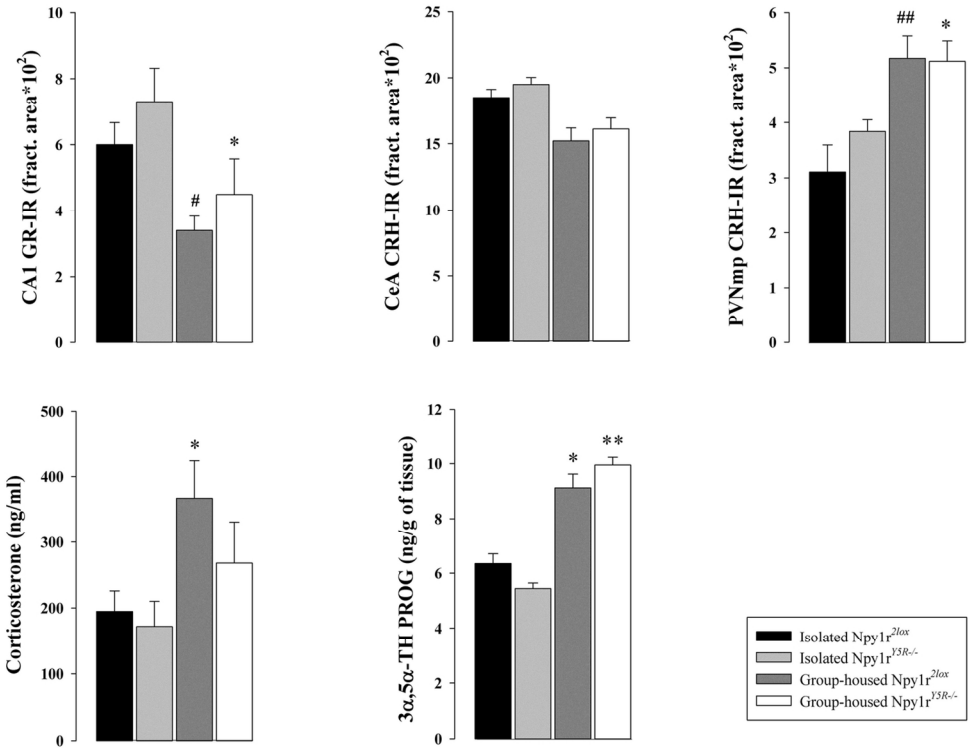
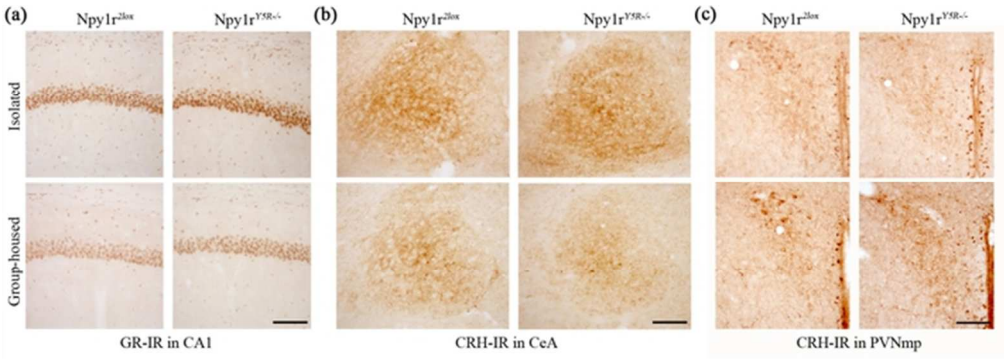


119x83mm (300 x 300 DPI)



127x96mm (300 x 300 DPI)



60x21mm (300 x 300 DPI)