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Conditional Inactivation of Neuropeptide Y Y1 Receptors Unravels the Role of Y1 and Y5 Receptors Coexpressing Neurons in Anxiety

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Abstract

Background The Y1 receptor (Y1R) and Y5 receptor (Y5R) for neuropeptide Y share similar actions in the regulation of anxiety. Previously demonstrated that conditional removal of the Y1R during postnatal development in the forebrain excitatory neurons leads to higher anxiety, increased hypothalamus-pituitary-adrenocortical axis activity, and decreased body growth rate in male mice raised by foster mothers that exhibit high levels of maternal care. In the present study, we used the same conditional system to analyze the specific contribution to emotional behavior and stress response of the Y1R coexpressed with the Y5R.

Methods Using the Cre-loxP recombination system, we investigated anxious behavior, spatial memory, and metabolic functions of conditional knockout mice in which the inactivation of the Npy1r gene was induced in the Y5Rs expressing neurons of juvenile mice (Npy1rY5R−/−).

Results Npy1rY5R−/− mice show increased anxiety-related behavior but no changes in hypothalamus-pituitary-adrenocortical axis activity or in body weight growth, independently of gender and mouse strain used as foster mothers. Also, Npy1rY5R−/− mice of both genders display increased spatial reference memory in the Morris water maze test.

Conclusions The results suggest that neuropeptide Y Y1R differentially expressed in the limbic system regulates anxiety and stress responses via distinct neurochemical circuits. In addition, we provide the first experimental genetic evidence that the Y1Rs coexpressed with the Y5R are involved in retention of spatial memory in male and female mice.

Key words: Behavioral flexibility; conditional knockout mice; Cre-loxP system; GABA; hypothalamus-pituitary-adrenocortical axis; spatial memory.

Introduction

Neuropeptide Y (NPY) is widely distributed in the central nervous system, where it is involved in the regulation of several biological functions, including emotionality and stress reactions, energy balance, and cognition 1, 2 and 3. In humans, NPY haploinsufficiency is correlated with brain responses to emotional and stress challenges and to anxiety (4). In rodents, injection of NPY into the third ventricle or into the limbic system reduces both anxiety and stress responses 5, 6 and 7. In the brain, NPY interacts with a family of G-protein–coupled receptors that includes the Y1 (Y1R), Y5 (Y5R), and Y2 receptors (the last one considered to function mainly as a presynaptic receptor on NPYergic terminals) 8 and 9.

Pharmacologic and genetic studies suggest that NPY induces anxiolytic effects via activation of the Y1Rs in amygdala, hippocampus, and locus coeruleus 10, 11, 12 and 13. We showed more recently that conditional inactivation of Npy1r gene in limbic excitatory neurons of Npy1rrfb (rfb = reduced forebrain expression) mice increases anxiety level and hypothalamus-pituitary-adrenocortical (HPA) axis activity and decreases body weight growth. Differences in phenotype between Npy1rrfb conditional mutants and their control littermates became apparent only when both genotypes were raised by dams exhibiting high levels of maternal care, suggesting that the Y1Rs expressed in limbic principal neurons are key targets of maternal care–induced programming of anxiety and energy homeostasis (10).

The Y5Rs and Y1Rs have overlapping function in regulating anxiety (14). The genes Npy1r and Npy5r are located on the same chromosome in humans and rodents, displaying an opposite transcriptional orientation and a partly overlapping gene structure (15). In rodents, the Y1R and Y5R are colocalized in several forebrain regions, including the basolateral amygdala (BLA) and hippocampal neurons 16 and 17. We hypothesized that the coordinated expression of the Y1R and Y5R might be required for the regulation of anxiety, spatial learning, and memory. In agreement, pharmacologic studies indicated that NPY induces anxiolytic effects via activation of the Y5R in the BLA. More recently, Domschke et al. (18) suggested that Npy5r gene variants may be associated with panic disorder, which is consistent with a role for the Y5R in anxiety disorders 14 and 19.

In the present study, we generated conditional knockout mice in which the inactivation of Npy1r was induced in Y5R-expressing neurons of adolescent mice (Npy1rY5R−/−). We achieved this by combining the gene targeted floxed Npy1r alleles (10) and the inducible Cre recombinase transgene (Cre) that is transcriptionally controlled by a bacterial artificial chromosome-encoded Npy5r promoter–driven tetracycline suppressible transactivator (tTA) (16). Our data reveal that conditional ablation of the Y1R in Y5R-expressing neurons results in increased anxiety-related behavior and improved spatial reference memory but no changes in basal or stress-activated HPA axis or body weight growth.

Methods and Materials

Animals

Mice were housed in groups of two to six in a temperature-controlled (22 ± 1°C) and humidity-controlled (50 ± 10%) room on a 12-hour light/dark cycle (8:00 am–8:00 pm) 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).

Generation of Npy1rY5R−/− Conditional Mutants

The region-specific inactivation of Npy1r gene in Y5R-expressing neurons in adolescent mice was achieved by using three different mouse lines: 1) Npy1r2lox, carrying a modified Npy1r allele in which two loxP sites flank the coding region of the Npy1r gene (10); 2) TgY5R-htTA/Y1RVenus, expressing a doxycycline (Dox)-sensitive, synthetic transcription factor (tTA) under the control of the Npy5r promoter (16); and 3) TgLC1, encoding a tTA inducible Cre transgene (20) (Figure 1A). Both loxP sites are DNA targets for Cre, which catalyzes site-specific recombination between the loxP sites and the removal of the Npy1r coding region (21). First, we generated two mouse lines, Npy1r2lox/TgY5R-htTA/Y1RVenus mice and Npy1r2lox/TgLC1 mice, which were inter-crossed providing offspring that were used as control animals (summarized as Npy1r2lox mice) as well as offspring Npy1r2lox/TgY5R-htTA/Y1RVenus/LC1 that contained all genetic elements necessary for the inactivation of the Npyr1 gene specifically in Y5R-containing neurons (summarized as Npy1rY5R−/− mice) (Figure 1B).

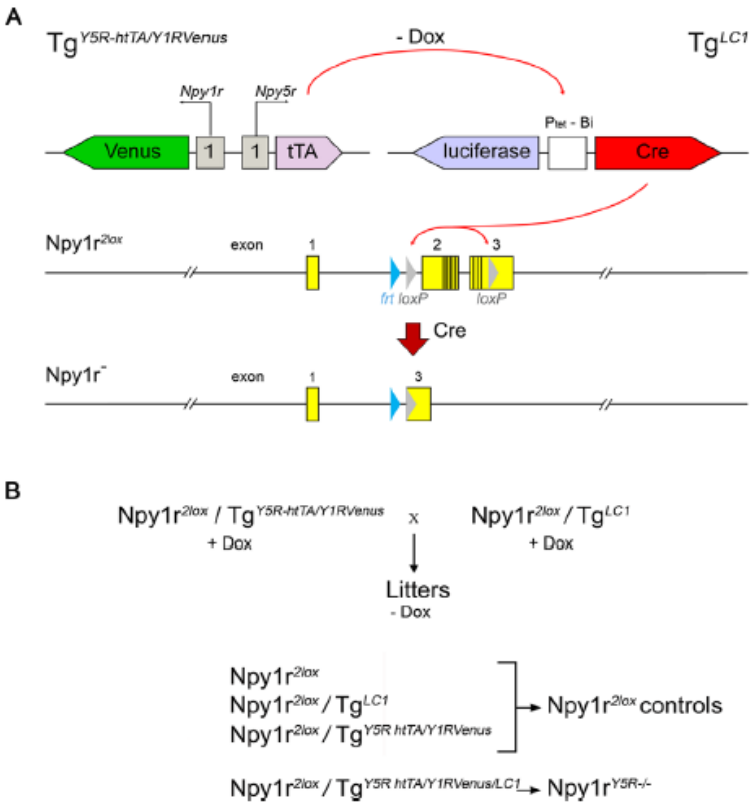

Figure 1. Generation of Npy1r$^{Y5R-/-}$ mutants. **(A)** Diagram depicting the interaction of the different genetic components. After doxycycline (Dox) removal at postnatal day (P) 0, the *Npy5r* promoter-driven Dox-sensitive, synthetic transcription activator (tTA) activates transcription of the transgene Tg^{LC1}, inducing Cre recombinase (Cre) expression in neurons containing the Y5 receptor (Y5R) subtype. Cre interacts with *loxP* sites in the gene-targeted Npy1r^{2lox} alleles (10) and removes the Npy1r^{2lox} coding region leading to the inactivation of the *Npy1r* gene (Npy1r−). *Frt* and *loxP* sites are in blue and gray triangles, respectively; exons are in open boxes, coding regions are in gray boxes; and transmembrane spanning codons are in black boxes. **(B)** By mating the compound transgenic mice Npy1r^{2lox}/Tg$^{Y5R-htTA/Y1RVenus}$ and Npy1r^{2lox}/Tg^{LC1} under Dox treatment, pups with four different genotypes were generated and found in a Mendelian ratio. At the day of birth (P0), the litters were transferred to Dox-naïve foster mothers to induce the Cre-mediated *Npy1r* gene inactivation in Y5R-containing neurons of Npy1r^{2lox}/Tg$^{Y5R-htTA/Y1RVenus/LC1}$ (Npy1r$^{Y5R-/-}$). Littermates comprising Npy1r^{2lox}/Tg$^{Y5R-htTA/Y1RVenus}$, Npy1r^{2lox}/Tg^{LC1}, and Npy1r^{2lox} genotypes were used as controls (Npy1r^{2lox} controls).

Long-term treatment of mothers with Dox (50 mg/L in drinking water, 1% sucrose; Sigma-Aldrich, Milano, Italy) prevents early Npy1r gene inactivation by an efficient suppression of tTA-dependent Cre expression 10 and 22. The Dox withdrawal at birth, by fostering litters to Dox-naïve dams at postnatal day (P) 0, slowly activates tTA, which is specifically expressed in Y5R-containing neurons. Activated tTA now induces Cre expression and, subsequently, Npy1r gene inactivation in Npy1rY5R−/− mice that is fully achieved between P45 and P50 (16) (Figure 1A,B and Figure S1 in Supplement 1). Mice (4–11 litters) were fostered at P0 to Dox-free FVB/J dams and analyzed between P60 and P90. To investigate whether early maternal environment may unmask the phenotype of Npy1rY5R−/− conditional mutants, mice from four to nine litters were fostered at P0 to Dox-free C57BL/6J dams exhibiting poor levels of maternal care compared with FVB/J dams (10).

Restraint Stress

The method used for restraint stress can be found in Supplemental Methods and Materials in Supplement 1.

Behavior

Open field (OF) and elevated plus maze (EPM) (to test anxiety and locomotor activity) were performed between P65 and P70 from 8:00–10:00 am (OF) and 5:00–7:00 pm (EPM). The Morris water maze (MWM) to test spatial memory was performed around P90 from 2:00–4:00 pm in an independent cohort of mice. Data were recorded automatically from the digitized image by using a computerized video tracking software. Detailed information can be found in Supplemental Methods and Materials in Supplement 1.

Body Weight and Food Intake

Body weight of mice housed in groups was measured twice a week from P30 to P90. For food intake and poststarvation refeeding test, mice were housed individually in cages starting at P30. At P70, food intake was measured every 24 hours for 5 days, and then mice were starved for 30 hours. After this period, food consumption was again measured 3 hours after refeeding and once a day for the subsequent 5 days.

Histologic Examination

Methods used for immunostaining, in situ hybridization, and quantification analysis can be found in Supplemental Methods and Materials in Supplement 1.

Real-Time Polymerase Chain Reaction

Methods used for real-time polymerase chain reaction and primer sequences can be found in Supplemental Methods and Materials in Supplement 1.

Serology

Methods used for serum collection and corticosterone analysis (radioimmunoassay) can be found in Supplemental Methods and Materials in Supplement 1.

Data Analysis

Three-way analysis of variance for repeated measures was used to compare mean body weight over time and food intake. All the other quantitative results were analyzed by two-way or three-way analysis of

variance, followed by Newman-Keuls Test, Bonferroni test for multiple comparisons, or Student t test when indicated. All data are expressed as mean ± SEM, and the level of statistical significance was set at $p < .05$.

Results

Verification of the Region-Specific and Temporal-Specific Npy1r Gene Inactivation in Npy1rY5R−/− Mice

Using the TgY5RitTA⁄Y1RVenus/LC1 mice, we previously demonstrated that Npy1r gene promoter-directed Venus and Npy5r promoter-directed tTA colocalize in several telencephalic and diencephalic structures, including cerebral cortex, hippocampus, amygdala, and hypothalamus (16). We expected the strongest decrease of Y1R expression in the hypothalamic and forebrain regions, similar to the reduction induced in the limbic structures of Npy1rrfb mice using the α-calcium/calmodulin-dependent protein kinase II (α-CamKII) promoter-controlled, Dox-attenuated Cre expression for postnatal inactivation of the Npy1r gene in excitatory neurons (10).

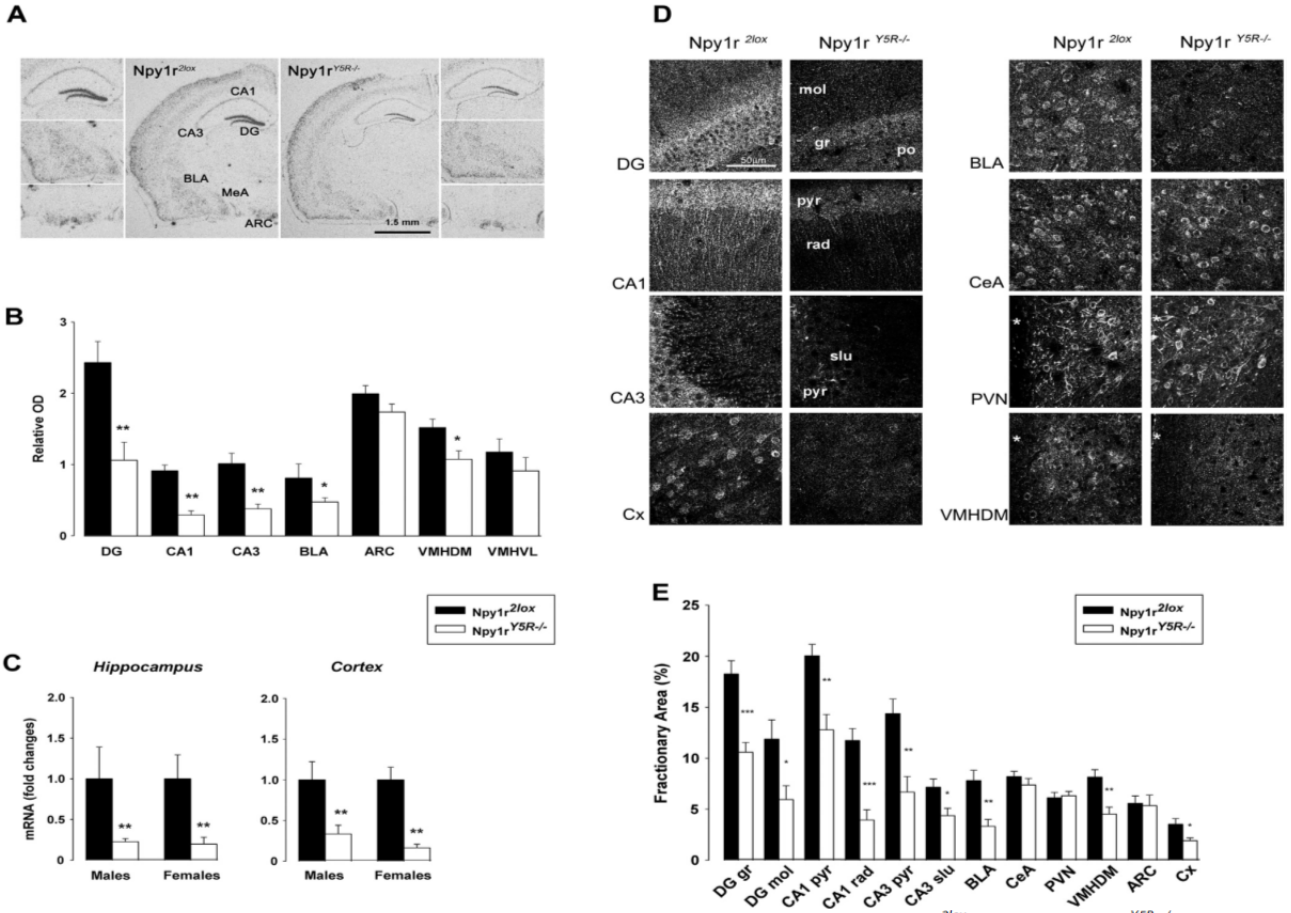

Figure 2. Expression of *Npy1r* messenger RNA (mRNA) and Y1 receptor (Y1R) peptide in the brain of Npy1r^{2lox} control and Npy1r$^{Y5R-/-}$ conditional mutants. **(A)** Representative autoradiograms of in situ hybridization of *Npy1r* mRNA on brain coronal sections from Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ male mice. **(B)** Quantitative signal intensity (OD) analysis of in situ hybridization showed a significant decrease of *Npy1r* mRNA expression in the hippocampus (DG granule cell layer and CA1 and CA3 pyramidal cell layer), in the BLA, and in the VMHDM nucleus of Npy1r$^{Y5R-/-}$ mice compared with control Npy1r^{2lox} littermates. Data are expressed as relative optical density and are the mean ± SEM from two independent experiments; $n = 5$. $^{**}p < .01$ and $^{*}p < .05$ vs. Npy1r^{2lox} mice (Student *t* test). (Scale bar = 1.5 mm.) **(C)** Npy1r$^{Y5R-/-}$ male and female mice showed lower *Npy1r* mRNA expression in the hippocampus and cerebral cortex compared with Npy1r^{2lox} siblings. Data are expressed as fold change variations after real-time polymerase chain reaction analysis and are mean ± SEM; $n = 3$–5 from three litters. Two-way analysis of variance revealed a significant effect of genotype for *Npy1r* in the hippocampus [$F_{1,15} = 9.03$, $p = .0089$] and in the cerebral cortex [$F1,8 = 25.94$, $p = .0009$]. $^{**}p < .01$ vs. male and female Npy1r^{2lox} mice, respectively (Bonferroni posttest). **(D)** The Y1R immunofluorescence confirmed the reduced Y1R expression in CA1, CA3, and DG; in the Cx; in the BLA; and in the VMHDM of Npy1r$^{Y5R-/-}$ mice compared with Npy1r^{2lox} littermates. The Y1R immunosignal was similar in the PVN and in the CeA. (Scale bar = 50 μm.) **(E)** Semiquantitative analysis of Y1R immunostaining showed a significant decrease of Y1R in the hippocampus (DG molecular and granule cell layers, CA1 and CA3 pyramidal cell layer, CA1 stratum radiatum, and CA3 stratum lucidum), in the BLA, in the VMHDM nucleus, and in the Cx of Npy1r$^{Y5R-/-}$ mice compared with control Npy1r^{2lox} littermates. Data are expressed as fractional area (%) and are mean ± SEM from three independent experiments; $n = 5$–11. $^{***}p < .001$, $^{**}p < .01$, and $^{*}p < .05$ vs. Npy1r^{2lox} mice (Student *t* test). ARC, arcuate nucleus; BLA, basolateral amygdala; CA1, CA1 stratum pyramidale; CA3, CA3 stratum pyramidale; CeA, central amygdala; Cx, cerebral cortex; DG, dentate gyrus; gr, granular cell layer; Mea, medial amygdala; mol, molecular layer; po, polymorphic layer; PVN, paraventricular nucleus; pyr, pyramidal cell layer; rad, stratum radiatum; slu, stratum lucidum; VMHDM, ventromedial hypothalamic nucleus, dorsomedial part; VMHVL, ventromedial hypothalamic nucleus, ventrolateral part; *third ventricle.

Conditional Cre-mediated inactivation of the Npy1r gene was first verified in Npy1rY5R−/− mice by semiquantitative in situ hybridization. A significant reduction of Npy1r messenger RNA (mRNA) expression was 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 Npy1rY5R−/− mice compared with their control littermates (Figure 2A,B). In the hypothalamus, Npy1rY5R−/− conditional mutants also displayed a significantly lower Npy1r mRNA expression in the dorsomedial part of the ventromedial nucleus compared with Npy1r2lox siblings (Figure 2A,B). As expected, no significant differences were observed in the hypothalamic arcuate nucleus where no colocalization of Venus and Cre reporter gene was previously observed (16). Npy1r mRNA in the paraventricular nucleus (PVN) was below the detection limit of the method and could not be measured.

In situ analysis was confirmed by real-time polymerase chain reaction. Npy1r mRNA was decreased in the hippocampus and in the cerebral cortex of Npy1rY5R−/− mice compared with control littermates of both genders (Figure 2C). The Npy1r gene expression profile was analyzed by immunofluorescence staining of coronal brain sections using an anti-Y1R antibody (Figure 2D). Semiquantitative analysis of Y1R immunoreactivity (IR) revealed a significant decrease of the receptor protein in the cerebral cortex, the DG (molecular and granule cell layers), the CA1 (stratum pyramidale and radiatum), the CA3 (stratum pyramidale and lucidum), the BLA, and the dorsomedial part of the ventromedial nucleus of Npy1rY5R−/− male mice compared with their control littermates (Figure 2E). No significant differences were observed in the central amygdala (CeA), the PVN, and the arcuate nucleus hypothalamic nuclei (Figure 2E).

Y1R and Y5R Coexist in Both GABAergic and Glutamatergic Neurons of the BLA

After postnatal Cre activation, the Npy1r gene was selectively removed in some brain areas of Npy1rY5R−/− mice, but residual Y1R still could be detected (Figure 2D,E and Figure S1 in Supplement 1). The residual Y1R is due to incomplete removal of the Npy1r gene in Y5R-expressing cells and to some nonoverlapping activities of the Npy1r and the Npy5r promoters (16), assuming that the Venus-induced and tTA-induced Cre expression reflects the endogenous Npy1r and the Npy5r promoter activity, respectively.

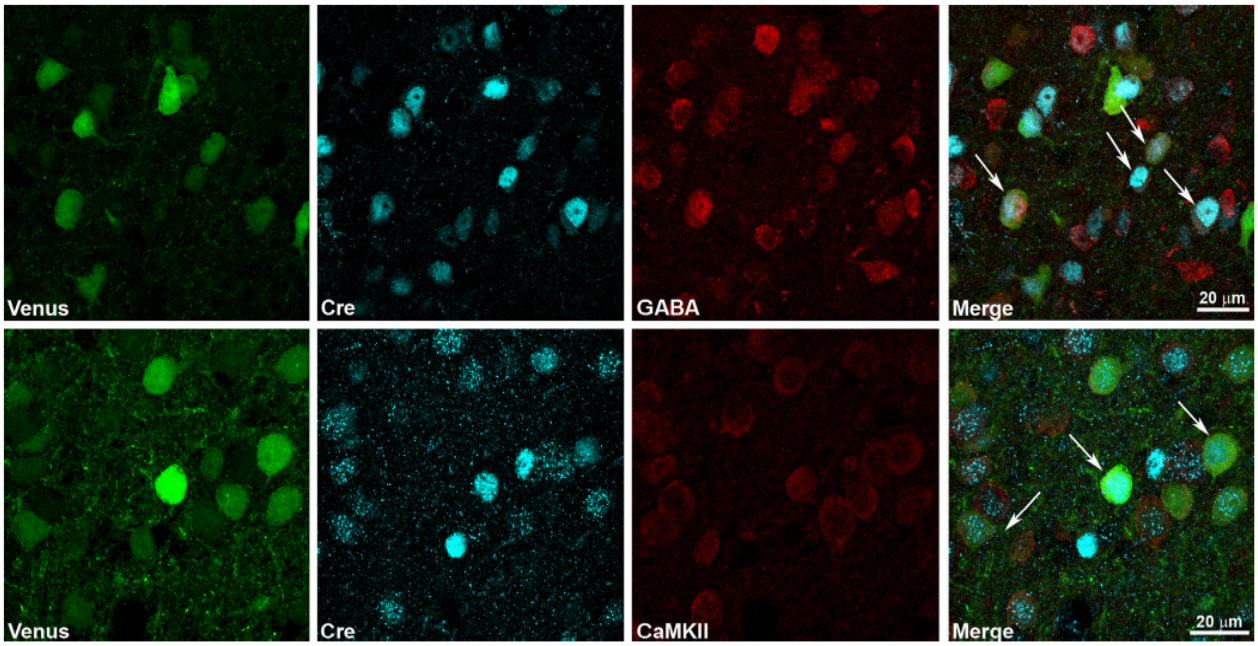

Figure 3. Colocalization of Y1 receptor (Y1R), Y5 receptor (Y5R), and gamma-aminobutyric acid (GABA) or α-calcium/calmodulin-dependent protein kinase II (α-CamKII) in the basolateral amygdala. Confocal images showed the distribution of Venus fluorescence, Cre recombinase (Cre) immunostaining, and GABA immunostaining or α-CamKII immunostaining in neurons of the basolateral amygdala. Merged images show the degree of colocalization of Venus, Cre, and GABA or α-CamKII as indicated by arrows. (Scale bar = 20 µm.)

To analyze in detail the predicted colocalization of Y1R and Y5R in interneurons and principal cells of the BLA, we monitored Venus fluorescence (Npy1r promoter) and Cre recombinase (Npy5r promoter) and gamma-aminobutyric acid (GABA) and α-CamKII (glutamate) immunoreactivities in coronal brain sections from Npy1rY5R−/− mice using confocal microscopy. The number of Y5Rs (Cre-positive cells) was higher and only partly overlapping with Y1Rs (Venus-positive cells). The analysis of sections from different animals (four per mouse) suggests that ≈50% of GABA immunofluorescent cells coexpress Venus and Cre, whereas only a subpopulation (≈25%) of glutamatergic α-CamKII-IR cells are positive for Venus-IR and Cre-IR (Figure 3).

Analysis of the Phenotype of Npy1rY5R−/− Mice

The removal of Y1Rs from α-CaMKII-expressing neurons in the limbic system of juvenile mice was correlated with decreased body weight growth, increased activity of the HPA axis, and increased anxiety (10). We analyzed whether the conditional inactivation of the Npy1r gene in Y5R-expressing neurons of the forebrain of adolescent Npy1rY5R−/− mice was also affecting these physiologic and behavioral parameters. Additionally, because it was described that transgenic rats overexpressing hippocampal NPY show signs of spatial memory impairments (23), we analyzed the Npy1rY5R−/− mice in the MWM test.

Conditional Inactivation of Y1R in Y5R-Expressing Neurons Does Not Affect Physiologic Functions

Conditional deletion of the Y1R in Y5R-expressing cells had no effect on the HPA axis activity of Npy1rY5R−/− mice under basal conditions, independent of gender. This lack of effect was demonstrated by measuring 1) the density of corticotropin-releasing hormone immunoreactive fibers and cell bodies in the CeA and in the medial parvocellular division of the PVN, 2) the density of glucocorticoid receptor (GR) immunoreactive neurons in the CA1, 3) the fold changes of GR mRNA in the whole hippocampus, and 4) the serum levels of corticosterone (Figure S2 and Table S1 in Supplement 1). The exposure to 30 minutes of restraint stress significantly increases corticosterone serum levels and GR-IR nuclear expression in the CA1 in both male and female control mice and conditional mutants, but no differences were observed between the two phenotypes (Figure S2 and Table S1 in Supplement 1). Similarly, conditional Npy1r gene inactivation failed to affect body weight growth (Figure 4A) and food intake (Figure 4B) of Npy1rY5R−/−, independent of gender.

Conditional Inactivation of Y1R in Y5R-Expressing Neurons Increases Anxiety

Npy1rY5R−/− mice showed increased anxietylike behavior as determined by a significantly lower frequency of entries and time spent in the open arms of the EPM (Figure 5A) and less time and shorter distance traveled in the center of the OF (Figure 5B) compared with Npy1r2lox siblings. The effect was similar in males and females and independent of locomotor activity (OF, total distance traveled [m], males, Npy1r2lox = 4.8 ± .34 and Npy1rY5R−/− = 4.5 ± .31, and females, Npy1r2lox = 5.2 ± .40 and Npy1rY5R−/− = 4.4 ± .22; EPM, total entries [no.], males, Npy1r2lox = 32 ± 3.5 and Npy1rY5R−/− = 28 ± 2.1, and females, Npy1r2lox = 31 ± 2.8 and Npy1rY5R−/− = 27 ± 2.5]. The anxiety-like behavior of Npy1rY5R−/− mice is significantly correlated with the Npy1r mRNA expression in hippocampal CA1 pyramidal cell layers and, to a lower extent, in CA3 and in the BLA, suggesting that the degree of reduced expression of the Y1R in the limbic system can predict the level of anxiety in the individual animals (Figure S3 in Supplement 1).

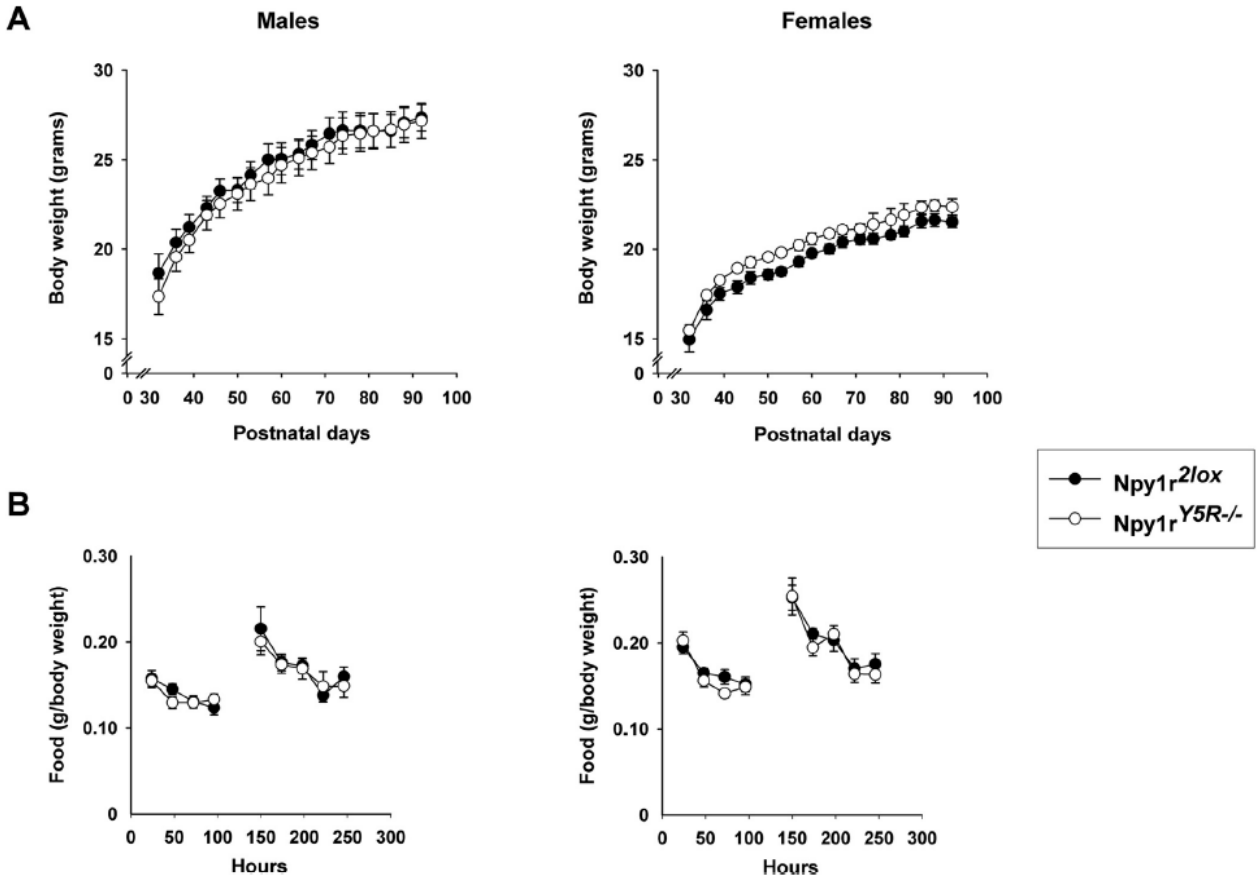

Figure 4. Body weight growth and food intake. **(A)** Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ male and female mice showed a similar body weight growth curve throughout the 3-month period of monitoring. **(B)** Food intake was similar between Npy1r^{2lox} controls and Npy1r$^{Y5R-/-}$ mice during the 4 days before 30-hour fasting, for 3 hours after refeeding, and for the subsequent 4 days. Data are mean ± SEM; n = 5–13 from six to eight litters.

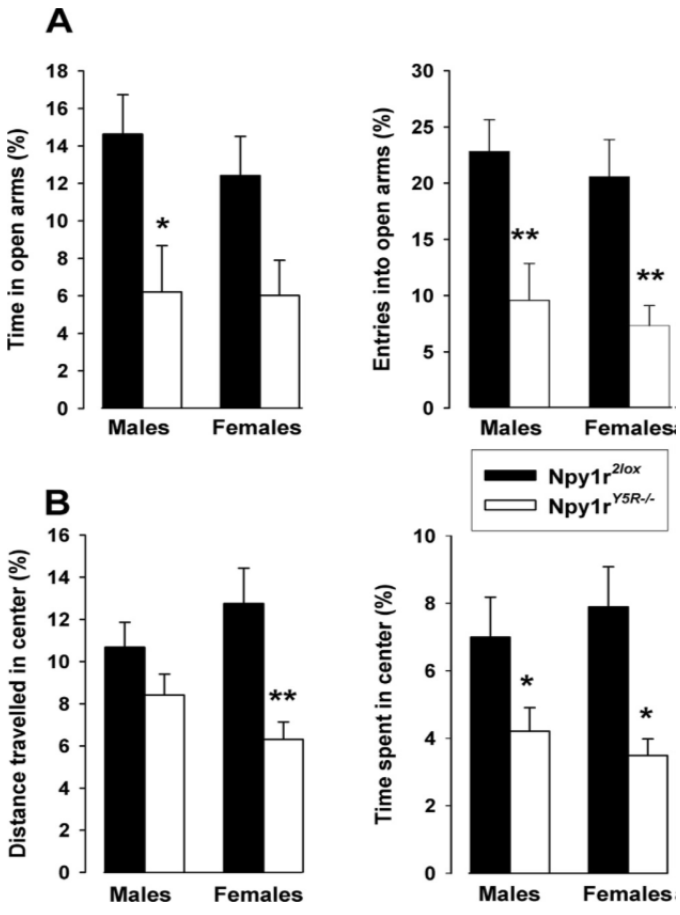

Figure 5. Anxiety-like behavior in the elevated plus maze and open field. Npy1r$^{Y5R-/-}$ mice showed higher anxiety levels in the elevated plus maze (**A**) and open field (**B**) tests compared with Npy1r^{2lox} mice, independent of gender. Data are mean ± SEM; n = 12–17 from 9–11 litters. For elevated plus maze, two-way analysis of variance revealed a significant effect of genotype for percent of time in open arms [$F_{1,55} = 10.13$, $p < .005$] and for percent of entries in open arms [$F_{1,55} = 23.78$, $p = .00001$]. $^{**}p < .01$ and $^{*}p < .05$ Npy1r$^{Y5R-/-}$ versus male and female Npy1r^{2lox} mice, respectively (Newman-Keuls test). For open field, two-way analysis of variance revealed a significant effect of genotype for percent of time spent in the center [$F_{1,47} = 15.32$, $p < .001$] and for percent of distance traveled in the center [$F_{1,47} = 14.98$, $p < .001$]. $^{**}p < .01$ and $^{*}p < .05$ Npy1r$^{Y5R-/-}$ versus male and female Npy1r^{2lox} mice, respectively (Newman-Keuls test).

Conditional Inactivation of Y1R in Y5R-Expressing Neurons Does Not Affect Learning but Increases Spatial Reference Memory

In the acquisition phase, Npy1r2lox and Npy1rY5R−/− mice showed similar decline of latency to find the hidden platform over 4 days of training indicating normal acquisition of spatial reference memory (Figure 6A). In the probe trial (conducted 24 hours after the last training trial), no significant differences in latency to reach the platform area were observed between Npy1rY5R−/− and Npy1r2lox mice independent of gender (latency [sec], males, Npy1r2lox = 7.9 ± 1.3 and Npy1rY5R−/− = 9.0 ± 2.5, and females, Npy1r2lox = 13 ± 2.3 and Npy1rY5R−/− = 13 ± 5.0; n = 9–16). Npy1rY5R−/− male and female mice showed a significantly higher percentage of time spent in the target zone (Figure 6B). Npy1rY5R−/− mice demonstrated a stronger preference for the probe quadrant because they spent more time in the target zone (north east) compared with the cumulative time spent in the no target zones (south east, south west, north west) (Figure 6C).

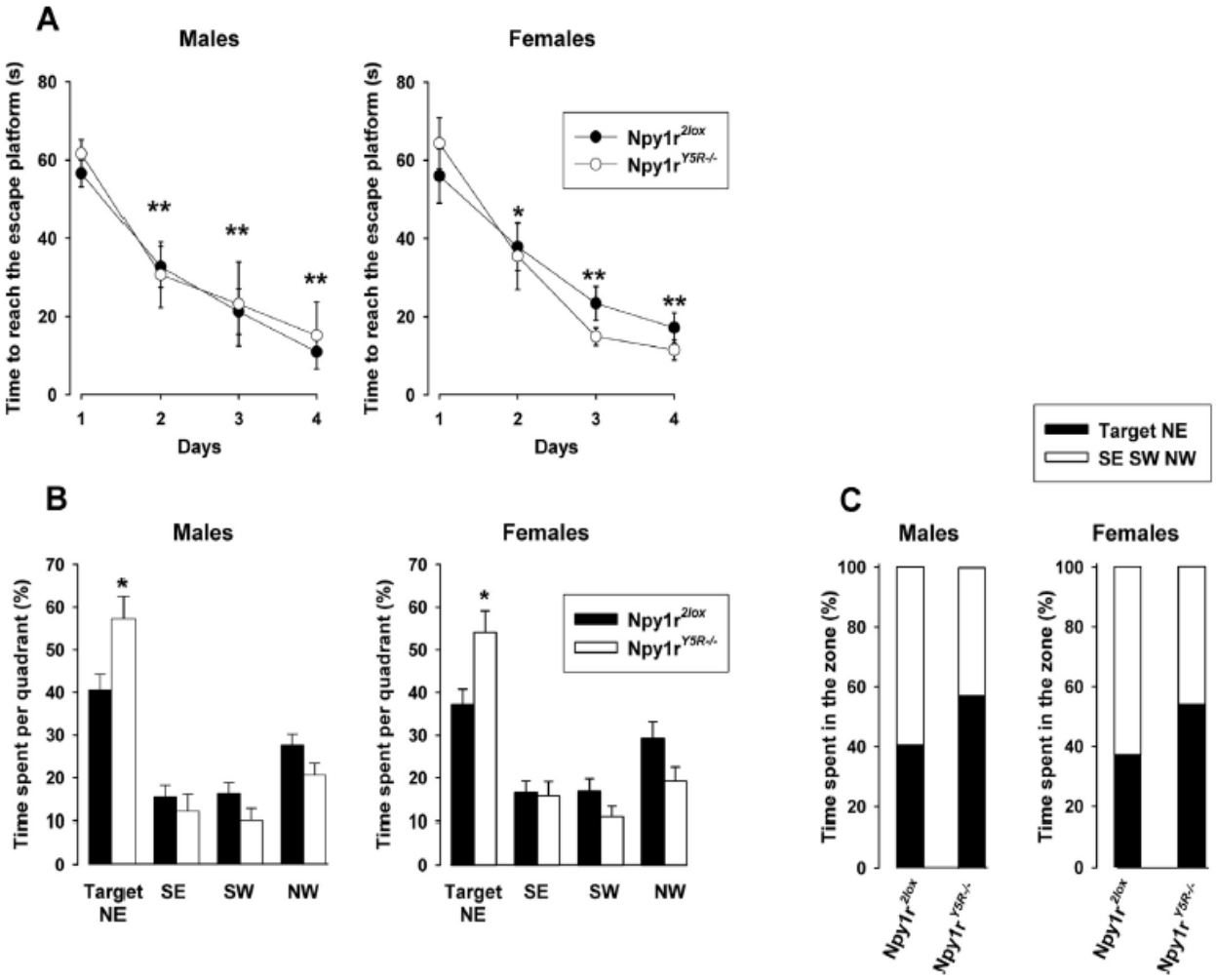

Figure 6. Spatial learning and memory retention in the Morris water maze. Npy1r$^{Y5R-/-}$ mice showed higher percentage of time spent in the target zone compared with Npy1r^{2lox} mice, independent of gender. (**A**) Acquisition phase. No significant difference in escape time was observed between Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice each day. Data are mean ± SEM; $n = 9$–16 from four to nine litters. After 4 days of training, the time to reach the escape platform for all groups decreased, indicating normal spatial learning memory. Two-way analysis of variance for repeated measures revealed a significant effect of days [$F_{3,138} = 63.15$, $p = .000$]. *$p < .05$ and **$p < .01$ compared with the time to reach the escape platform at day 1. (**B**) Probe trial. After training, the platform was removed from the north east quadrant (NE, target zone) where it was originally placed. The y axis indicates the percentage of total time spent in one specific quadrant. Data are mean ± SEM; $n = 9$–16 from four to nine litters. Two-way analysis of variance revealed a significant effect of genotype for percentage of total time spent in NE target quadrant [$F_{1,46} = 16.376$, $p = .0002$] and for percentage of time spent in south west (SW) [$F_{1,46} = 5.206$, $p = .0272$] and north west (NW) [$F_{1,46} = 7.369$, $p = .00093$] quadrants. *$p < .05$ Npy1r$^{Y5R-/-}$ vs. Npy1r^{2lox} mice (Newman-Keuls Test). (**C**) Percentage of time spent in NE target and no target (south east [SE], SW, NW) zones. Data are mean ± SEM; $n = 9$–16 from four to nine litters. Two-way analysis of variance revealed a significant effect of genotype for percentage of total time spent in target and no target quadrants [$F_{1,46} = 16.39$, $p = .0002$].

We also measured the path length after mice reached the target zone in the probe trial (24). An increased path length outside the target zone, after reaching the original location of the platform, would suggest increased flexibility in an attempt to look for a new location of the platform. On the contrary, longer path length in the target zone would suggest persistency possibly related to increased anxiety. Both female and male Npy1rY5R−/− mice displayed a significantly shorter path length outside the target zone after reaching the original position occupied by the platform compared with their control siblings, whereas Npy12lox mice showed an increased path length outside the target zone, independent of gender (Figure 7A,B).

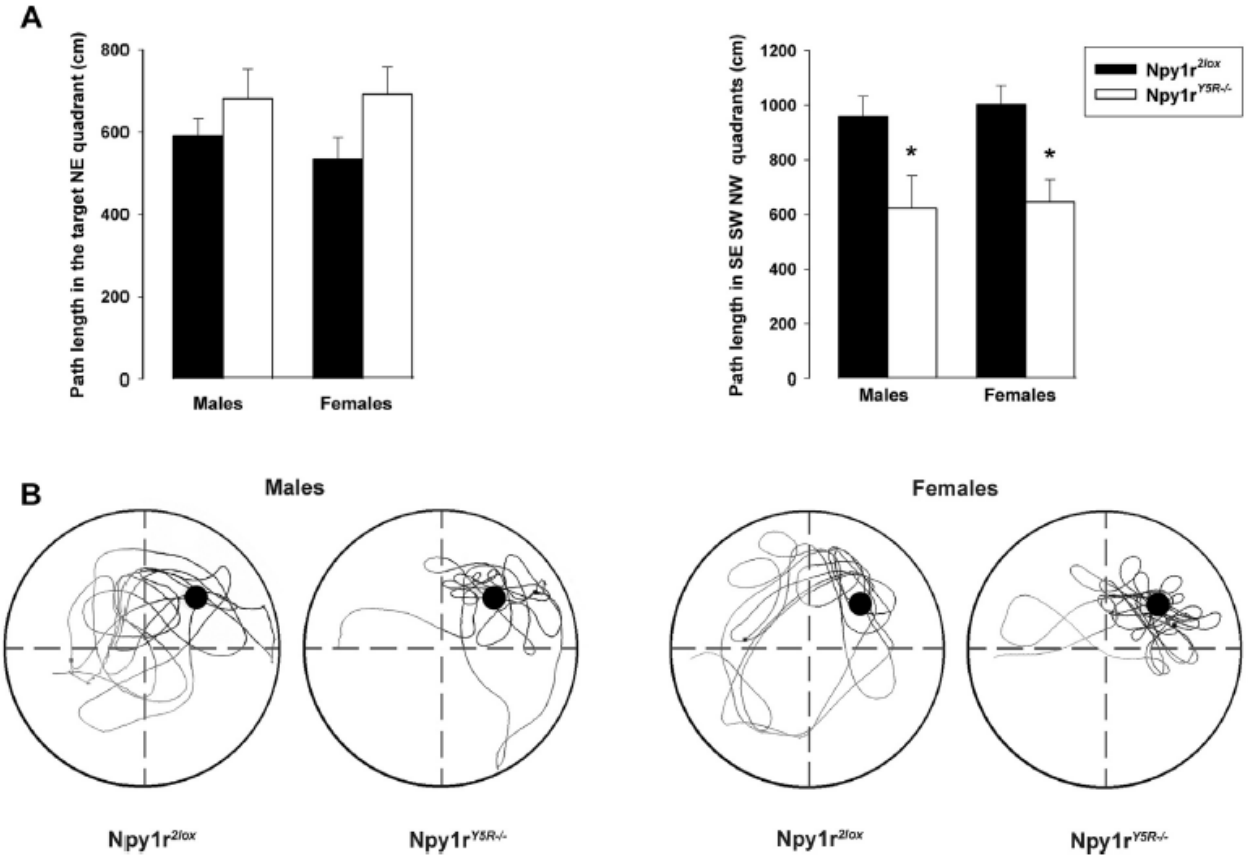

Figure 7. Path length in Morris water maze. Measures of the total distance covered (path length) by Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ mice after they reached the target zone in the probe trial. **(A)** Npy1r$^{Y5R-/-}$ mice showed longer path length (cm) in the target north east (NE) quadrant and shorter path length in south east (SE), south west (SW), and north west (NW) quadrants compared with Npy1r^{2lox} mice, independent of the gender. Data are mean ± SEM; n = 9–16 from four to nine litters. Two-way analysis of variance revealed a significant effect of genotype for path length in NE target quadrant [$F_{1,46} = 5.058$, $p = .0293$] and no target (SE, SW, NW) zones [$F_{1,46} = 17.97$, $p = .00011$]. *$p < .05$ vs. Npy1r^{2lox} mice (Newman-Keuls Test). **(B)** Representative swim paths of Npy1r^{2lox} and Npy1r$^{Y5R-/-}$ male and female mice in the Morris water maze. The examples show that Npy1r$^{Y5R-/-}$ male and female mice cover the longest path length in the target NE quadrant, and Npy1r^{2lox} male and female mice cover the longest path length in the SE, SW, and NW quadrants.

Differences in Phenotypes Between Npy1rY5R−/− and Npy1r2lox Mice Are Independent of Maternal Care Received

We previously demonstrated that conditional inactivation of the Npy1r gene in the limbic excitatory neurons of adult Npy1rfb mice leads to different behavioral and physiologic consequences depending on early maternal environment. We compared the differences in phenotype between Npy1r2lox and Npy1rY5R−/− mice within animals from the same litter raised either by FVB/J dams, displaying high levels of maternal care, or by C57BL/6J dams, showing low maternal behavior versus the adopted pups (10).

As previously reported (10), low levels of maternal care has long-lasting impact on C57BL/6J-fostered Npy1r2lox control mice that show lower body weight, higher anxiety level, and corticotropin-releasing hormone IR in the PVN compared with FVB/J-fostered Npy1r2lox mice from the same litter (Table 1). Npy1rY5R−/− mice showed increased anxiety-related behavior in the EPM and improved spatial reference

memory in the MWM compared with Npy1r2lox siblings, demonstrating that the physiologic and behavioral phenotype of conditional mutants is unaffected by the maternal care received.

Table 1. Comparison of Phenotypes of $Npy1r^{2lox}$ and $Npy1r^{Y5R-/-}$ Male Mice Fostered to FVB/J and C57BL/6J Dams

Foster Mother Strain	FVB/J		C57BL/6J	
Phenotype	$Npy1r^{2lox}$	$Npy1r^{Y5R-/-}$	$Npy1r^{2lox}$	$Npy1r^{Y5R-/-}$
BW (Grams)	25.6 ± .57	27.2 ± 1.0	23.2 ± .63[a]	23.5 ± .67[a,b]
EPM (% Time in Open Arms)	14.6 ± 2.1	6.2 ± 2.5[c]	6.5 ± 2.0[d]	1.2 ± .71[d,e]
PVN CRH-IR (Fractional Area × 10^{-3})	7.8 ± .8	7.0 ± 1.1	11 ± 1.1[f]	13 ± 1.9[f,g]
MWM (% of Time in NE Target Zone)	41.01 ± 2.8	59.57 ± 4.8[h]	42.52 ± 3.4	55.5 ± 5.2[i]

Body weight, anxiety-like behavior (elevated plus maze), corticotropin-releasing hormone immunoreactivity in the paraventricular nucleus of the hypothalamus, and spatial memory retention (Morris water maze) of $Npy1r^{Y5R-/-}$ conditional mutant male mice and their control littermates fostered to FVB/J and C57BL6/J dams at postnatal day (P) 0. Mice were analyzed at P60 (body weight), P65 (elevated plus maze), and P90 (Morris water maze and corticotropin-releasing hormone immunoreactivity). FVB/J-fostered $Npy1r^{2lox}$ control mice showed higher body weight, lower anxiety levels, and lower density of corticotropin-releasing hormone immunoreactive cell bodies in the paraventricular nucleus than the C57BL/6J-fostered $Npy1r^{2lox}$ controls, as previously reported (10). Data are expressed as mean ± SEM (body weight, $n = 7–13$ from five to six litters; elevated plus maze, $n = 8–14$ from eight to nine litters; paraventricular nucleus corticotropin-releasing hormone immunoreactivity, $n = 3–5$ from two litters; Morris water maze, $n = 8–18$ from four to six litters).

BW, body weight; CRH-IR, corticotropin-releasing hormone immunoreactivity; EPM, elevated plus maze; MWM, Morris water maze; NE, north east; PVN, paraventricular nucleus.

BW: Two-way analysis of variance (ANOVA) revealed a significant effect of strain of foster mother [$F_{1,35} = 19.3$, $p < .0001$]. [a]$p < .05$ vs. FVB fostered $Npy1r^{2lox}$ mice; [b]$p < .01$ versus FVB fostered $Npy1r^{Y5R-/-}$ mice (Newman-Keuls Test).

EPM: Two-way ANOVA revealed a significant effect of strain of foster mother and genotype [$F_{1,46} = 9.71$, $p = .0032$; $F_{1,46} = 8.81$, $p = .0047$, respectively]. [c]$p < .05$ and [d]$p < .01$ vs. FVB fostered $Npy1r^{2lox}$ mice (Newman-Keuls Test); [e]$p < .05$ vs. C57BL6/J fostered $Npy1r^{2lox}$ mice (Student *t* test).

PVN CRH-IR: Two-way ANOVA revealed a significant effect of strain of foster mother [$F_{1,13} = 16.9$, $p = .0012$]; [f]$p < .05$ vs. FVB fostered $Npy1r^{2lox}$ mice; [g]$p < .05$ vs. FVB fostered $Npy1r^{Y5R-/-}$ mice (Newman-Keuls Test).

MWM: Two-way ANOVA revealed a significant effect of genotype [$F_{1,49} = 15.4$, $p = .00027$]; [h]$p < .01$ vs. FVB fostered $Npy1r^{2lox}$ mice and [i]$p < .05$ vs. C57BL6/J fostered $Npy1r^{2lox}$ mice.

Discussion

To analyze the specific contribution of Y1Rs coexpressed with Y5Rs on NPY mediated physiologic functions, we generated conditional Npy1r knockout mice (Npy1rY5R−/−) exhibiting inactivation of Y1Rs selectively in Y5R-containing neurons of adult animals. In situ hybridization, quantitative real-time polymerase chain reaction, and immunofluorescence revealed reduced Y1R mRNA and protein levels in the cerebral cortex; in the CA1, CA3, and DG of the hippocampus; in the BLA; and in the hypothalamic area dorsomedial part of the ventromedial nucleus. Npy1rY5R−/− mice showed increased anxiety and higher behavioral persistency possibly related to improved spatial reference memory compared with control littermates.

The anxiogenic effect of specific inactivation of the Y1R in Y5R-containing neurons became apparent in the EPM and OF tests. In the EPM, Npy1rY5R−/− mice showed a lower frequency of entries and spent significantly less time in the open arms. In the OF, Npy1rY5R−/− conditional mutants of both genders spent less time and traveled a shorter distance in the center of the arena compared with their control littermates. These results suggest that neurons containing both Y1Rs and Y5Rs play a major role in the control of anxious behavior, without affecting HPA axis activity, as shown by substantial identical levels of serum corticosterone and corticotropin-releasing hormone expression in CeA and PVN and hippocampal GR-IR and mRNA in control and Npy1rY5R−/− mice under basal conditions and after 30 minutes of restraint stress.

By using the same conditional system, we generated knockout mice (Npy1rrfb) exhibiting reduced levels of Y1Rs in adult limbic excitatory neurons (10). Npy1rrfb and Npy1rY5R−/− targeted mice display a few similarities and differences. Both Npy1rrfb and Npy1rY5R−/− male mice displayed anxiety-like behavior in EPM and OF; however, Npy1rrfb male mice, but not Npy1rY5R−/− mice, showed increased HPA activity. Npy1rrfb male mice showed a significant decrease of body weight growth compared with their control littermates, whereas no changes were observed in Npy1rY5R−/− male mice. When we used the α-CamKII promoter-controlled Cre to remove Y1Rs from principal cells of the forebrain of juvenile mice (Npy1rrfb),

the development of a behavioral and physiologic impairment compared with wild-type littermates was gender-specific and inversely correlated with maternal care (10). On the contrary, the phenotype of Npy1rY5R−/− mice was largely independent of gender and maternal care. These differences between phenotypes of Npy1rY5R−/− and Npy1rrfb mice may be explained by the different regional and cellular inactivation pattern of the Npy1r gene.

Limbic stress effector pathways relay through basal forebrain, hypothalamic, and brainstem neurons that innervate the medial parvocellular division of the PVN to modulate the HPA axis. This neuronal circuitry more recently has been proposed to be involved in long-lasting impact of maternal care on stress resilience (25). The hippocampus is an important component of this circuit controlling anxiety-related behaviors and stress responses and seems to inhibit the HPA axis through glutamate-GABA connections (26). We previously postulated that in Npy1rrfb mice, the selective inactivation of Y1Rs in principal excitatory neurons of hippocampus lead to increased HPA axis activity via the glutamatergic output, which possibly depends on early maternal conditions (10).

Both Npy1rY5R−/− and Npy1rrfb mice display a significant reduction of Npy1r mRNA and protein in the hippocampal CA1 and CA3 pyramidal and DG granule cell layers compared with their control littermates. However, comparison of Cre recombinase expression and Npy1r inactivation pattern in limbic system of these conditional mutants demonstrates that Npy1rY5R−/− mice, but not Npy1rrfb mice, show a significant decrease of Npy1r mRNA and protein in the BLA. Our data also indicate that in the BLA ≈50% of GABA-IR cells coexpress the Y1R and Y5R, whereas a small population of α-CamKII-positive neurons (≈25%) colocalize with both NPY receptor subtypes, suggesting that the conditional inactivation of the Npy1r gene in Npy1rY5R−/− mice might occur mainly in GABAergic neurons of the BLA. This finding indicates that the loss of Y1Rs also on the cellular level is different between Npy1rY5R−/− and Npy1rrfb mice, which may account for the diverse Npy1rY5R−/− and Npy1rrfb mice expressed phenotypes.

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. In the BLA, GABAergic interneurons have strong synaptic control over its principal neurons, the pyramidal cells (27). The presence of the Y1Rs on this population of interneurons could have profound effects on the synchronous activity of the BLA (28).

These results suggest that the circuitry by which NPY exerts its anxiolytic action is likely to involve more neuronal pathways. Molosh et al. (29) suggested more recently that two distinct, cyclic adenosine monophosphate-mediated intracellular mechanisms are operative in the Y1R-mediated control of N-methyl-D-aspartate and GABAA receptor functions in the BLA. Previous studies showed a striking dichotomy between CeA and BLA in the NPY regulation of behavioral, autonomic, and endocrine responses during stress, suggesting that different components of stress response not only are elicited through a wide range of pathways but also are differentially regulated (7). Consistent with these studies, we now suggest that a similar dichotomy may exist between the BLA GABAergic interneurons and hippocampal excitatory neurons in Y1R-mediated regulation of anxiety and the HPA axis that may explain the differences between phenotypes of Npy1rrfb and Npy1rY5R−/− mice.

In the present study we also demonstrate that conditional inactivation of Npy1r increased spatial reference memory. In the MWM, Npy1rY5R−/− mice showed similar latencies to reach the platform as control Npy1r2lox mice but a significant increase of time spent in the target zone, 1 day after 4 days of training. This finding suggests that the deficit in memory retention, previously observed in transgenic rats

overexpressing NPY in the hippocampus (23), might be mediated by the Y1R coexpressed in Y5R-containing neurons, providing novel evidence for a role of the Y1R in retention of spatial memory. The higher persistence of Npy1rY5R−/− mice in the target zone might also be related to lower behavioral flexibility, which would be consistent with the high anxiety profile of these animals. The behavioral flexibility requires that the animal does not persist in choosing a wrong solution (i.e., perseverating in the area where the platform was before) and rapidly modifies its behavior to search for a new solution 22 and 30. The underlying mechanisms allowing such adaptive behavior belong to a behavioral inhibition system, whose functions are controlled, in particular, by hippocampal formation 31, 32 and 33. However, further experiments are necessary to test this issue specifically by using a target-reversal design.

Previous studies have shown that GRs participate in the negative feedback inhibition of the HPA axis and modulate learning and memory (34) and that forebrain GR overexpression leads to cognitive deficits (35). Our findings demonstrate that hippocampal GR mRNA and protein expression and corticosterone levels were not affected by the conditional inactivation of Npy1r in Y5R-expressing neurons. It is unlikely that dysregulated hippocampal GRs accounts for the improvement of spatial reference memory observed in Npy1rY5R−/− conditional knockout mice.

It was postulated more recently that Y1R and Y5R colocalization results in receptor heterodimerization and in enhanced or altered function of several physiologic systems (36). The anxious phenotype of Npy1rY5R−/− mice might be due to both the conditional inactivation Npy1r and the altered Y5R functions. Domschke et al. (18) suggested that a coding variant and an intronic single nucleotide polymorphism in Npy5r, found to be associated with panic disorders, might directly or indirectly downregulate Y5R expression, contributing to an increased vulnerability to anxiety states. In line with these observations, it is reasonable to expect that conditional inactivation of Npy5r in Y1R-coexpressing neurons might similarly result in increased anxiety-related behavior and improved spatial reference memory. Although the interaction mechanisms between Y1Rs and Y5Rs require further evaluation, their synergistic action in the regulation of emotional behavior and behavioral flexibility may have new therapeutic potentials for human anxiety.

In conclusion, we established a genetic tool to inactivate spatially and temporally Npy1r gene in neurons coexpressing both the Y1R and the Y5R. Our analysis demonstrates that Npy1rY5R−/− mutants show a strong anxious phenotype that is not associated with increased HPA axis activity; this phenotype is independent of gender and early maternal care, and we suggest it might be mechanistically linked to inactivation of the Y1R in GABAergic interneurons of the BLA. In addition, we provide experimental genetic evidence that Y1Rs coexpressed with Y5Rs are involved in spatial reference memory and possibly in behavioral flexibility in male and female mice. Together, these data strongly support the concept that NPY Y1R, acting on distinct limbic neurotransmitter systems, may play different physiologic roles in the regulation of anxiety and stress response. If we consider our findings in the context of human studies, showing a direct relationship between NPY and interindividual variation in emotion and stress resilience (4), our data suggest a potential strategy of novel therapeutics for various disorders related to anxiety and stress.

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