Regulatory functions of limbic Y1 receptors in body weight and anxiety uncovered by conditional knockout and maternal care

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Neuropeptide Y (NPY) plays an important role in stress, anxiety, obesity, and energy homeostasis via activation of NPY-Y1 receptors (Y1Rs) in the brain. However, global knockout of the *Npy1r* gene has low or no impact on anxiety and body weight. To uncover the role of limbic Y1Rs, we generated conditional knockout mice in which the inactivation of the *Npy1r* gene was restricted to excitatory neurons of the forebrain, starting from juvenile stages (Npy1r^{rfb}). Npy1r^{rfb} mice exhibited increased anxiety and reduced body weight, less adipose tissue, and lower serum leptin levels. Npy1r^{rfb} mutants also had a hyperactive hypothalamic–pituitary–adrenocortical axis, as indicated by higher peripheral corticosterone and higher density of NPY immunoreactive fibers and corticotropin releasing hormone immunoreactive cell bodies in the paraventricular hypothalamic nucleus. Importantly, through fostering experiments, we determined that differences in phenotype between Npy1r^{rfb} and Npy1r^{2lox} mice became apparent when both genotypes were raised by FVB/J but not by C57BL/6J dams, suggesting that limbic Y1Rs are key targets of maternal care-induced programming of anxiety and energy homeostasis.

foster mother | maternal behavior | Cre-LoxP system

Neuropeptide Y (NPY) is widely distributed in the CNS, where it is involved in the regulation of anxiety, stress reactions, energy balance, circadian rhythms, and cognition (1–3). Clinical studies suggest that NPY plays an important role in the response to stress and in psychiatric disorders (4). In humans, NPY haploinsufficiency is correlated with characteristic brain responses to emotional and stress challenges and with trait anxiety (5). Intracerebroventricular injection of NPY reduces both anxiety- and stress-related behavior in several animal models, an effect that is primarily mediated by Y1 receptors (Y1Rs) expressed in amygdala, hippocampus, and locus coeruleus (6–9). The implications of a role of endogenous NPY in acting via Y1R to control emotionality, mood, and stress reactions have been probed with Y1R-selective antagonists and antisense oligonucleotides (1). NPY exerts its anxiolytic-like effect in the brain via interactions with the hypothalamic–pituitary–adrenocortical (HPA) axis and corticosteroids. Indeed, a functional antagonism between NPY and corticotropin releasing hormone (CRH) has been demonstrated in various CNS nuclei along the stress/anxiety circuits (10).

In addition to its crucial role in emotional behavior, NPY potently stimulates feeding, reduces energy expenditure, and induces obesity via the activation of Y1R expressed in the hypothalamus (1). However, global *Npy1r* gene knockout mice showed only minor deficiencies in energy homeostasis, feeding, and anxiety (11–14).

To study the function of Y1R expressed in the limbic system and to exclude effects induced by the *Npy1r* gene inactivation in early development, we restricted the ablation of Y1R to excitatory neurons in the postnatal forebrain of mice by using the Cre-*loxP* system (Fig. 1*A*). In addition, because early postnatal environment can modulate NPY levels (15), gene-targeted pups were reared by two different strains of foster mothers. Our study indicates that conditional inactivation of the *Npy1r* gene in principal neurons of the forebrain of male mice led to increased anxiety level and lower body weight, both of which depend on early maternal conditions.

Results

Generation of Npy1r^{rfb} Conditional Mutant Mice. Inactivation of the *Npy1r* gene in the juvenile forebrain was achieved in mice carrying gene-targeted floxed *Npy1r* alleles (Fig. S1) and an inducible Cre recombinase (Cre) transgene [Fig. 1; ref. 16). Cre expression in these mice is controlled by a doxycycline (Dox) sensitive, synthetic transcriptional activator (tTA) (Fig. 1*A*) and, thus, chronic Dox treatment of pregnant females from conception prevented early *Npy1r* inactivation by efficient suppression of the tTA-dependent Cre expression (Fig. 1*B*). Litters were fostered to Dox-naïve dams (Fig. 1*C*), thus activating tTA, which was transgenically expressed in principal neurons of the forebrain via the α-calcium/calmodulin-dependent protein kinase II (α–CamKII) promoter (16–18). In absence of Dox, tTA induced Cre expression and, subsequently, *Npy1*r gene inactivation in juvenile Npy1r^{2lox}/Tg$^{\alpha CamKII\text{-}tTA/LC1}$ mice (Npy1r^{rfb} mice; rfb, reduced forebrain expression). Cre-mediated recombination of floxed cellular target alleles in excitatory neurons of the forebrain is complete between postnatal days (P)35 and 50 (17, 19).

Because anxiety, stress response, and NPY levels (Fig. 1*C*) (15, 20) are affected by early postnatal environment and variations in maternal care, we used foster mothers from two different mouse strains, FVB/J or C57BL/6J, which show different quality of maternal cares (21). More specifically, C57BL/6J mice, which are the background strain from which Npy1r^{2lox} mice are derived, were reported to show very low arched-back nursing compared with several inbred strains (22). By following this breeding scheme, we obtained four groups of mice: Npy1r^{2lox} control mice and Npy1r^{rfb} littermates, both nursed by either C57BL/6J or by

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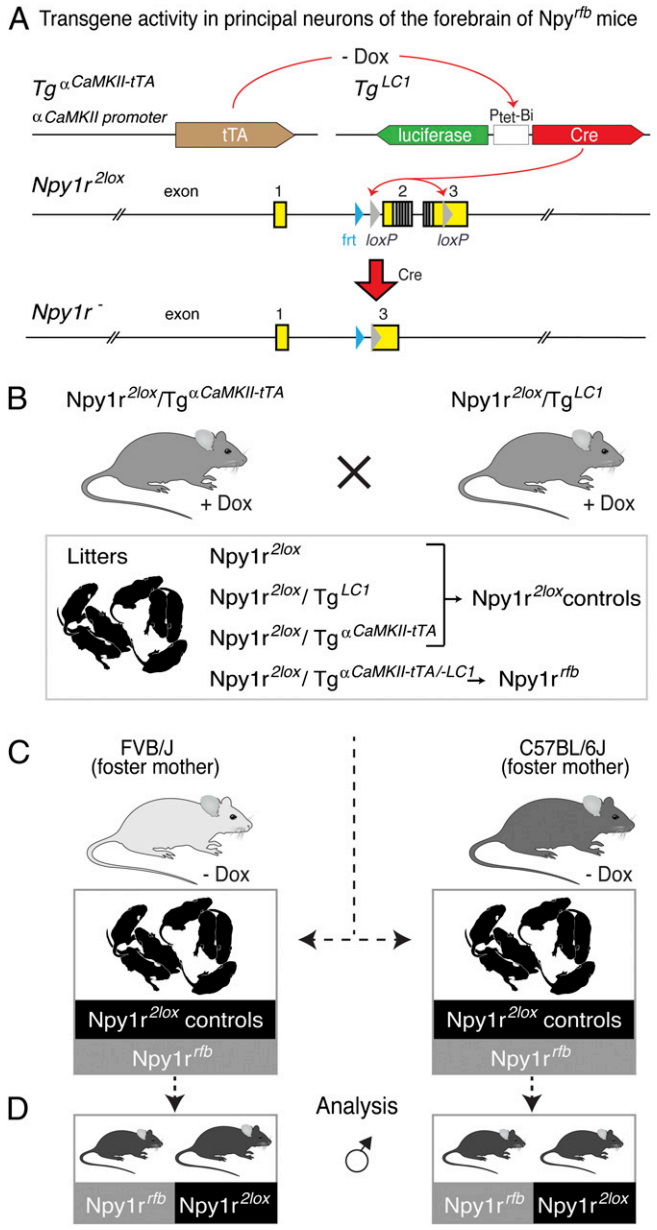

Fig. 1. Generation of Npy1r^{rfb} mutants and Npy1r^{2lox} control cohorts used for the analysis. (*A*) Diagram depicting the interaction of the different genetic components: After Dox removal, the αCamKII promoter-driven tTA activates transcription of the transgene *Tg^{LC1}*, thereby inducing Cre expression in excitatory neurons of the forebrain. The Cre recombinase interacts with *loxP* sites in the gene-targeted *Npy1r^{2lox}* alleles and removes the *Npy1r^{2lox}* (*SI Materials and Methods*) coding region leading to the inactivation of the *Npy1r* gene (*Npy1r^{-}*). Frt and *loxP* sites are in blue and gray triangles, respectively; exons in open boxes, coding regions in gray boxes; black boxes, transmembrane spanning codons. (*B*) By mating the compound transgenic mice Npy1r^{2lox}/Tg$^{αCamKII-tTA}$ and Npy1r^{2lox}/Tg^{LC1} under Dox treatment, pups with four different genotypes were generated and found in a Mendelian ratio. (*C*) At the day of birth [postnatal day (P)0], the litters were transferred to either C57BL/6J or FVB/J Dox naive foster mothers to induce the Cre-mediated *Npy1r* gene inactivation in the forebrain of Npy1r^{2lox}/Tg$^{αCamKII-tTA\ /LC1}$ mice (named herein Npy1r^{rfb}). Littermates comprising Npy1r^{2lox}/Tg$^{αCamKII-tTA}$, Npy1r^{2lox}/Tg^{LC1}, and Npy1r^{2lox} genotypes were used as controls (named herein Npy1r^{2lox} controls). (*D*) The comparative analysis of Npy1r^{rfb} and Npy1r^{2lox} controls was used to uncover the function of Y1R in the limbic system of mice that experienced differences in maternal care during the first three weeks of life, as indicated by increased body weight of adult Npy1r^{2lox} mice fostered to FVB/J dams compared with other littermates.

FVB/J foster mothers. All male mice were analyzed as adults after weaning (Fig. 1*D*).

Analysis of Maternal Behavior of FVB/J and C57BL/6J Foster Dams. Individual differences in maternal behavior of FVB/J and C57BL/6J dams toward fostered pups were characterized, in an independent cohort of mice, during the first postnatal week of life. Direct observation of mother–pup interactions revealed considerable variations in different forms of maternal behavior (Fig. 2*A*). The overall time spent nursing the fostered pups by FVB/J and C57BL/6J dams was not significantly different (Fig. 2 *A* and *B*). However, FVB/J dams spent more time crouching over the pups in the active form of nursing known as arched-back nursing (ABN) compared with C57BL/6J dams, which more frequently adopted a lying posture to nurse the pups (23, 24) (Fig. 2*B*). FVB/J foster mothers showed lesser self-grooming and nest building, ate more, and were less active than C57BL/6J dams (Fig. 2*A*). Litters reared by FVB/J dams displayed higher growth curves starting at P6 than C57BL/6J-reared litters (Fig. 2*C*).

Importantly, differences in maternal care cannot be attributed to the strain of pups because FVB/J dams displayed similar levels of maternal behavior toward fostered pups derived from FVB/J strain than toward Npy1r^{2lox}-fostered pups (C57BL/6J derived strain).

Region- and Temporal-Specific Npy1r Inactivation Depends on the Strain of the Adoptive Mother. Conditional Cre-mediated inactivation of *Npy1r* was first verified by semiquantitative in situ hybridization. In Npy1r^{rfb} mice fostered to FVB/J mothers, Cre recombination led to a significant reduction of *Npy1r* mRNA expression in the hippocampal CA1 and CA3 pyramidal and in the dentate gyrus (DG) granule cell layers, compared with their control littermates (Fig. 3 *A* and *B*).

When litters were raised by C57BL/6J mothers, Npy1r^{2lox} mice showed lower *Npy1r* mRNA in CA1, CA3, and DG than FVB/J-fostered Npy1r^{2lox} mice (Fig. 3 *A* and *B*), suggesting that limbic *Npy1r* expression depends on maternal care. More importantly, Npy1r^{rfb} mice fostered to C57BL/6J mothers did not show the expected down-regulation of *Npy1r* mRNA, possibly due to the already low *Npy1r* expression in C57BL/6J-fostered Npy1r^{2lox} mice (Fig. 3*B*).

The limbic *Npy1r* expression profile was confirmed by immunohistochemistry of coronal brain sections using an Y1R-specific

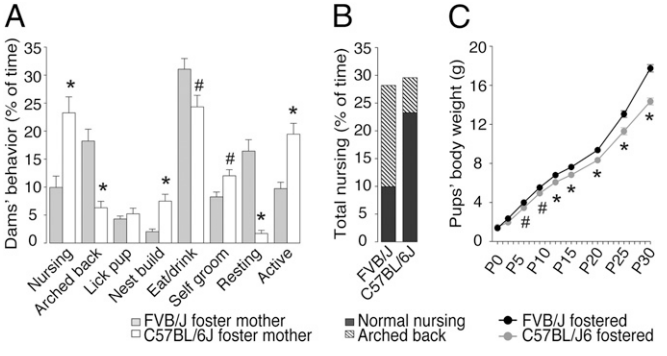

Fig. 2. Maternal behavior of FVB/J and C57BL/6J foster mothers. Average percent time spent by FVB/J and C57BL/6J dams on maternal behavior variables (*A*) and on total nursing (*B*) during postnatal days P1–P8. Data are the mean ± SEM from two independent experiments; n = 7–9. $^{*}P < 0.01$; $^{\#}P < 0.05$ by unpaired t test for independent samples. Average body weight of pups (6–8 litters) fostered to C57BL/6J and to FVB/J mothers is noted. Data are representative of two independent experiments. Two-way ANOVA revealed a significant effect of foster mother strain: [$F_{(1,12)} = 21.6$; $P < 0.001$] and of foster mother strain–days interaction: [$F_{(1,96)} = 20.3$; $P < 0.001$]. $^{*}P < 0.01$; $^{\#}P < 0.05$, by Newman–Keuls.

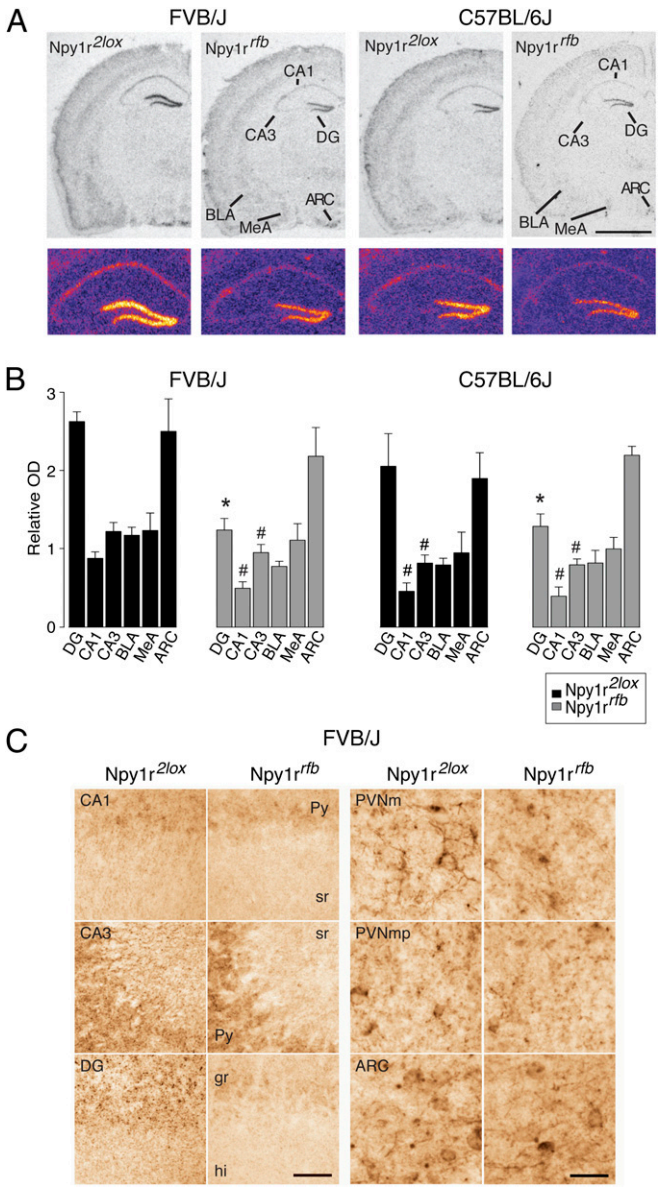

Fig. 3. Expression of *Npy1r* mRNA and Y1R peptide in the brain of control and conditional mutants raised by FVB/J and C57BL/6J dams. "FVB/J" and "C57BL/6J" refer to foster mother strain. (*A*) Representative autoradiograms of in situ hybridization of *Npy1r* mRNA on brain coronal sections of Npy1r^{2lox} and Npy1r^{rfb} mice fostered to FVB/J (*Left*) and to C57BL/6J (*Right*) dams. (Scale bar: 1.5 mm.) (*B Left*) Quantitative signal intensity (OD) analysis of in situ hybridization revealed the strongest significant decrease of *Npy1r* mRNA expression in CA1 and DG cell bodies of FVB/J fostered Npy1r^{rfb} mice compared with their control littermates. (*B Right*) Quantitative signal intensity (OD) analysis of in situ hybridization revealed no significant differences between C57BL/6J fostered Npy1r^{rfb} and Npy1r^{2lox} mice. A decrease of *Npy1r* mRNA expression was detected in the CA1, CA3, and DG of C57BL/6J fostered Npy1r^{2lox} compared with FVB/J fostered Npy1r^{2lox} mice (*Left*). (*A* and *B*) Data are expressed as optical density and are the mean ± SEM from two independent experiments; n = 3–4. Two-way ANOVA revealed a significant effect of genotype for DG [$F_{(1,10)} = 68.0$; $P < 0.001$], a significant effect of genotype and foster mother strain for CA1 [$F_{(1,10)} = 7.32$; $P < 0.05$ and $F_{(1,10)} = 8.37$; $P < 0.05$, respectively] and a significant effect of foster mother strain for CA3 [$F_{(1,10)} = 11.04$; $P < 0.01$]. $^{*}P < 0.01$ and $^{\#}P < 0.05$ versus Npy1r^{2lox} mice fostered to FVB/J dams, by Newman–Keuls. CA1, CA1 stratum pyramidale; CA3, CA3 stratum piramidale; DG, dentate gyrus; BLA, basolateral amygdala; CeA, central amygdala; MeA, medial amygdala. (*C*) The immunohistochemical anti-Y1R staining confirmed the reduced Y1R expression in cellular layers of DG and CA1 of FVB/J fostered Npy1r^{rfb} mice compared with their Npy1r^{2lox} littermates. (Scale bar: 50 μm.) The hypothalamic Y1R immunosignal was similar in the PVN [magnocellular (PVNm) and medial parvocellular (PVNmp)] division and in the arcuate (ARC) nuclei (Scale bar: 25 μm).

antibody (25, 26) (Fig. 3*C*). Y1R was reduced in the cellular layers of CA1, DG and, to a lower extent, CA3 of FVB/J-fostered Npy1r^{rfb} mice compared with control littermates, whereas it was unchanged in the amygdaloid nuclei as well as in the hypothalamic paraventricular (PVN) [magnocellular (PVNm) and medial parvocellular (PVNmp) division] and arcuate (ARC) nuclei (Fig. 3*C*), which did not express Cre recombinase (Fig. S2 *A* and *B*).

Consistent with the in situ hybridization data, C57BL/6J-fostered mice showed a lower Y1R immunosignal than that observed in FVB/J-fostered Npy1r^{2lox} mice (Fig. S3). These findings demonstrated that the strain of the foster mother influences the expression of limbic Y1R and suggested that conditional inactivation of *Npy1r* gene could lead to different behavioral and physiological consequences depending on early maternal environment. Thus, we expected the strongest phenotypic difference between Npy1r^{rfb} conditional mutants and Npy1r^{2lox} control littermates when fostered to FVB/J mothers, whereas any phenotypic effects between mutants and control littermates raised by C57BL/6J mothers should disappear.

Effect of Conditional *Npy1r* Inactivation on Anxiety-Related Behavior and Neuroendocrine Functions Depends on the Strain of the Adoptive Mother. ***Anxiety-related behavior.*** We used the elevated plus-maze (EPM) and the open field (OF) tests to investigate anxiety. In the EPM, Npy1r^{rfb} conditional mutants reared by FVB/J mothers showed a significantly lower frequency of entries and time spent in the open arms than their control littermates (Fig. 4*A*). In the OF, the same mice were less active and displayed significantly higher immobility in the center (Fig. 4*A*). These findings are conventionally interpreted as indicating increased anxiety (27).

In contrast, no difference in behavior emerged between mutant and control mice reared by C57BL/6J mothers in either the EPM or the OF tests (Fig. 4*A*). Notably, C57BL/6J-reared control mice showed a higher anxiety level than FVB/J-fostered controls, as demonstrated by the significantly lower frequency of entries and time spent in the open arms of the EPM and increased immobility in the center of the OF (Fig. 4*A*).

Neuroendocrine functions. Npy1r^{rfb} mice fostered to FVB/J dams displayed higher density of NPY immunoreactive fibers and CRH immunoreactive cell bodies in the PVNmp than their control littermates (Fig. 4*C*), which suggests increased central drive of the HPA-axis activity (25, 28). Consistently, basal serum corticosterone was higher in FVB/J-fostered mutant than control mice (Fig. 4*B*). Conversely, conditional *Npy1r* inactivation led to a lower density of CRH immunoreactive fibers in the central amygdala (CeA) of FVB/J-fostered mice (Fig. 4*D*).

No differences in corticosterone, NPY, or CRH immunoreactivity were observed between C57BL/6J-reared mutants and their control littermates (Fig. 4 *B–D*). Significantly, control mice fostered to C57BL/6J dams showed higher NPY and CRH immunoreactivity in the PVNmp, higher serum corticosterone levels, and lower CRH immunoreactive fibers in the CeA than FVB/J-fostered controls (Fig. 4 *B–D*).

Effect of Conditional Npy1r Inactivation on Body Weight, Food Intake, and Hormone Serum Levels Depends on the Strain of the Adoptive Mother. We observed that during postnatal development, between P41 and P48, Npy1r^{rfb} mice reared by FVB/J dams showed a slower body weight increase than FVB/J-reared controls (Fig. 5*A*). Limbic *Npy1r* may be involved in the lower growth curve of mice, considering that conditional Cre-mediated inactivation of the limbic *Npy1r* gene is induced in the same time window

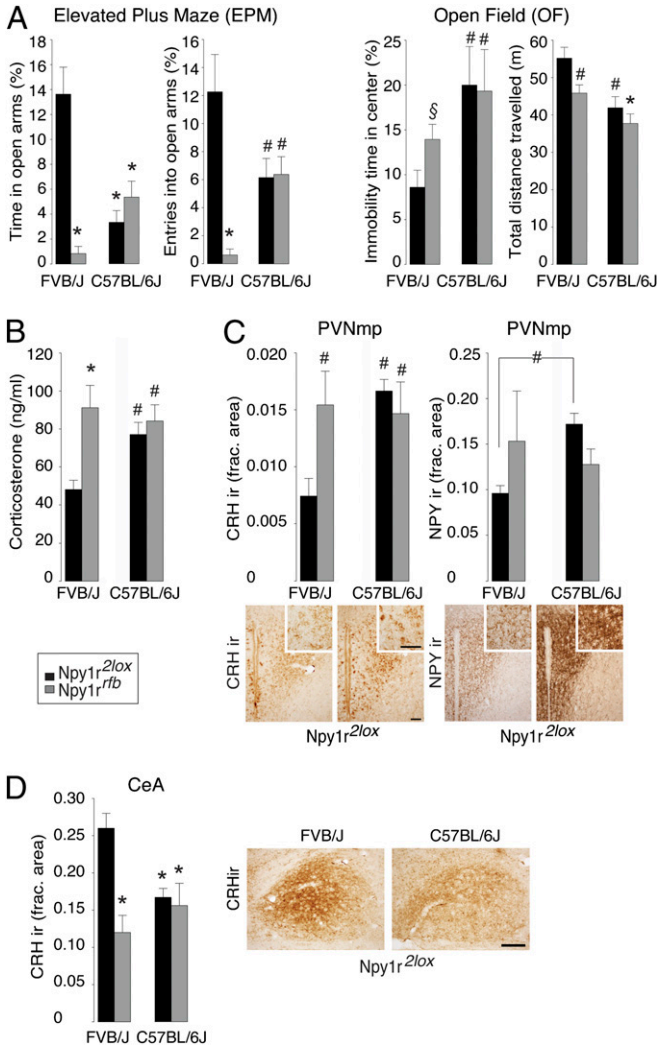

Fig. 4. Anxiety-like behavior, CRH, and neuroendocrine functions. (*A*) On the *x* axis is indicated the strain of the foster mother. FVB/J fostered Npy1r^{rfb} mice and C57BL/6J fostered mice, the latter independently of the genotype, showed higher anxiety level in the EPM and OF tests compared with FVB/J fostered Npy1r^{2lox} mice. Data are the mean ± SEM; n = 12–18 from seven to nine litters. (*A Left*) Two-way ANOVA revealed a significant effect of genotype, foster mother strain, and genotype–foster mother strain interaction for percent of time in open arms [$F_{(1,59)}$ = 14.0, $P < 0.001$; $F_{(1,59)}$ = 4.03, $P < 0.05$; $F_{(1,59)}$ = 26.4, $P < 0.001$, respectively], and genotype and genotype–foster mother strain interaction for percent of entries in open arms [$F_{(1,59)}$ = 10.8, P = 0.001; $F_{(1,59)}$ = 11.7, $P < 0.005$, respectively]. *$P < 0.01$ and $^{\#}P < 0.05$ versus Npy1r^{2lox} mice fostered to FVB/J mothers, by Newman–Keuls. (*A Right*) Two-way ANOVA revealed a significant effect of foster mother strain for percent of time of immobility in the center [$F_{(1,59)}$ = 4.99, $P < 0.05$], genotype and foster mother strain for total distance traveled [$F_{(1,59)}$ = 12.92, $P < 0.001$; $F_{(1,59)}$ = 11.67 $P < 0.005$, respectively]. *$P < 0.01$ and $^{\#}P < 0.05$ versus Npy1r^{2lox} mice fostered to FVB/J mothers. $^{\S}P$ = 0.052 versus Npy1r^{2lox} mice raised by FVB/J mothers, by Newman–Keuls. (*B*) FVB/J fostered Npy1r^{rfb} and C57BL/6J fostered mice of either genotype showed a significant increase of serum levels of corticosterone compared with FVB/J fostered Npy1r^{2lox} mice. Data are the mean ± SEM from three independent experiments (n = 9–15). Two-way ANOVA revealed a significant effect of genotype [$F_{(1,40)}$ = 7.41, $P < 0.05$]; genotype–foster mother strain interaction: $F_{(1,40)}$ = 3.88, P = 0.056. $P < 0.05$ versus Npy1r^{2lox} mice fostered to FVB/J mothers, by Newman-Keuls. (*C*) FVB/J fostered Npy1r^{rfb} mice and C57BL/6J fostered mice of either genotype showed increased density of CRH immunoreactive cell bodies and of NPY immunoreactive fibers in the medial parvocellular division of the paraventricular nuclei (PVNmp). Data are expressed as the mean fractional area ± SEM (n = 5–7 from three litters). (Scale bar: 50 μm; *Inset*, 25 μm.) CRH: Two-way ANOVA revealed a significant effect of genotype–foster mother interaction [$F_{(12,20)}$ = 5.9, $P < 0.05$]. *$P < 0.05$ versus Npy1r^{2lox} mice fostered to FVB/J mothers by Newman–Keuls. NPY: *$P < 0.05$ by Student's t test. (*D*) FVB/J fostered Npy1r^{rfb} mice and C57BL/6J fostered mice of either genotype showed decreased density of CRH immunoreactive fibers in the CeA. Data are expressed as the mean fractional area ± SEM (n = 5–7 from three litters). Two-way ANOVA revealed a significant effect of genotype and of genotype–foster mother strain interaction [$F_{(1,21)}$ = 12.0, $P < 0.005$; $F_{(1,21)}$ = 8.84, P = 0.01, respectively]. *$P < 0.01$ versus Npy1r^{2lox} mice fostered to FVB/J mothers by Newman–Keuls. (Scale bar: 50 μm.)

(Fig. S2 *A* and *B*). After induction at P41–P48, the difference in body weight persisted throughout a 7-mo period, when FVB/J-fostered Npy1r^{rfb} mice weighed ≈20% less than their control littermates (body weight (grams), Npy1r^{2lox} = 38 ± 3.0; Npy1r^{rfb} = 30 ± 1.2, n = 6; t: −2,747, $P < 0.05$). Decrease in body weight of mutant adult mice was associated with a significant decrease of weight of visceral and s.c. adipose fat pad (WAT) and of leptin serum levels (Fig. 5 *B* and *C*). Food intake (Fig. 5*C*) and locomotor activity [distance (meters), FVB/J-fostered mice: Npy1r^{2lox} = 878 ± 61 and Npy1r^{rfb} = 856 ± 54] were similar between controls and conditional mutants and, therefore, could be excluded as a primary cause of the different growth curves.

Mice reared by C57BL/6J dams exhibited lesser growth (Fig. 5*A*), leptin serum levels (Fig. 5*B*), and WAT weight (Fig. 5*C*) than FVB/J-fostered mice, independently of the genotype. Importantly, starting at P55–P60, the growth curve of FVB/J-fostered Npy1r^{rfb} mice overlapped with that of C57BL/6J-fostered mice (Fig. 5*A*). Overall, no significant differences were observed in food intake (Fig. 5*C*) or locomotor activity between FVB/J- and C57BL/6J-fostered littermates.

Discussion

To analyze limbic Y1R-mediated physiological functions, we generated conditional *Npy1r* knockout mice (Npy1r^{rfb}) exhibiting reduced levels of Y1Rs in the adult forebrain. Npy1r^{rfb} male mice showed increased anxiety, lower body weight, reduced adipose tissue, decreased serum leptin, and higher hypothalamic NPY and CRH expression levels. This phenotype of Npy1r^{rfb} mutants, however, became evident only in males reared by FVB/J, but not by C57BL6/J, foster mothers.

The anxiogenic effect of region-specific inactivation of the *Npy1r* gene was monitored in the OF and EPM tests. Npy1r^{rfb} mice displayed reduced exploration of the OF and higher immobility in the central area, suggestive of anxiety-induced "freezing" behavior (27). In the EPM, mutants showed a lower frequency of entries and spent significantly less time in the open arms. The finding of increased anxiety when the hippocampal Y1Rs are reduced is consistent with the anxiolytic effects that were described earlier in mice that overexpress virally transduced NPY in the hippocampus (8).

We found that limbic Y1R has a role not only in emotional behavior but also in the regulation of energy homeostasis. The growth of Npy1r^{rfb} mutants slowed down at approximately P40, which coincides with the maximal levels of *Npy1r* gene Cre-mediated inactivation. The lower body weight persisted through adulthood and was associated with lower white adipose tissue weight and leptin serum levels.

The exact function of forebrain-expressed Y1Rs in the control of energy balance remains unknown. Central administration of Y1R agonists increases food intake in rodents, an effect that is associated with stimulation of Y1R in the hypothalamus (1). It was reported that inactivation of NPY or Y2 receptors in the hypothalamus of adult mice induces, at most, small effects on appetite and transiently affects body weight, consistent with adaptation to homeostasis (29, 30). Conversely, our study indicates that the loss of limbic Y1Rs in adults cannot be compensated by such adaptive receptor specific mechanisms.

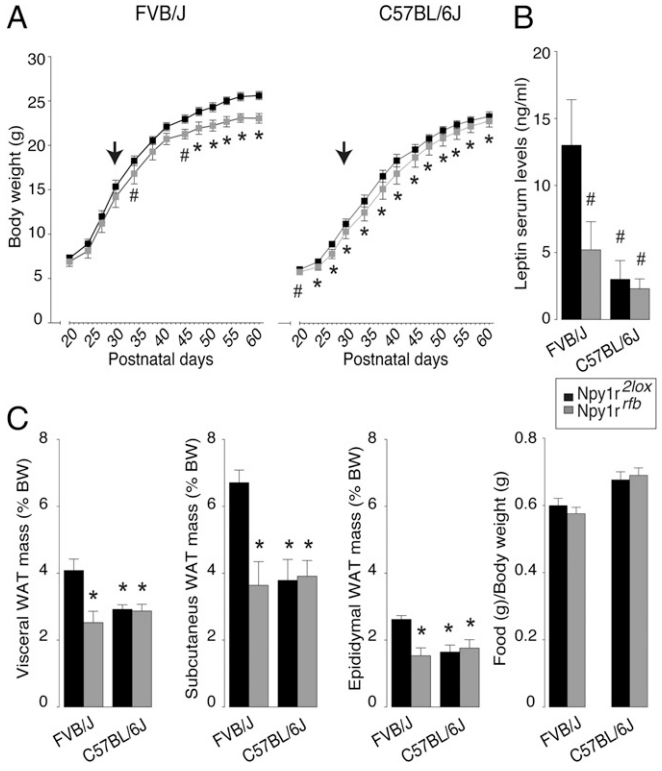

Fig. 5. FVB/J fostered Npy1r^{rfb} mice and C57BL/6J mice of either genotype showed lower growth rate (*A*), decreased leptin serum levels (*B*), and lower weight of visceral (mesenteric + perirenal + retroperitoneal), s.c. (inguinal + dorso-scapular), and epididymal white adipose tissue (WAT) (*C*) compared with FVB/J-fostered Npy1r^{2lox} mice. No significant differences were found for the daily food intake (*C*). Data are the mean ± SEM. Growth rate: Three-way ANOVA for repeated measurements revealed a significant effect of genotype [$F_{(1,57)} = 4.4$, $P < 0.05$], foster mother strain [$F_{(1,57)} = 21.3$, $P < 0.001$], and postnatal days– foster mother strain interaction [$F_{(12,\ 684)} = 10.8$, $P < 0.001$]; n = 13–16 from eight to nine litters. Leptin serum levels: Two-way ANOVA revealed a significant effect of foster mother strain [$F_{(1,31)} = 5.52$, $P < 0.05$]; n = 7–13 from two litters. WAT weight: Two-ways ANOVA revealed a significant effect of foster mother strain, genotype, and their interaction [visceral WAT: $F_{(1,13)} = 10.0$, 18.0, and 15.8, s.c. WAT: $F_{(1,13)} = 13.1$, 13.0, and 14.2, epididymal WAT: $F_{(1,13)} = 8.1$, 9.3, and 12.3, respectively; $P < 0.01$]; n = 4–7 from two litters. $^{*}P < 0.01$ and $^{\#}P < 0.05$ versus Npy1r^{2lox} fostered to FVB/J by Newman–Keuls test.

Conditional inactivation of limbic Y1R might decrease body weight gain by activation of the HPA axis. Limbic stress effector pathways relay through basal forebrain, hypothalamic, and brainstem neurons that, in turn, innervate the PVNmp to modulating the HPA axis. The hippocampus is an important component of neuronal circuitry controlling anxiety-related behaviors and stress responses and seems to inhibit the HPA axis through glutamate-GABA connections (31). Previous studies have shown that Y1Rs are associated with glutamate-positive and NPY-positive neurons in hippocampal subregions, providing the anatomical basis for the Y1R-mediated modulation of glutamate and NPY release (32, 33). Thus, we postulate that the selective inactivation of Y1Rs in principal excitatory neurons of hippocampus might stimulate HPA axis via the glutamatergic output.

However, given that hypothalamic and brainstem NPY neurons act as integrators between stress signals and the neuroendocrine response to stress (34, 35) and that NPY stimulates HPA axis via CRH neurons (25), the increase of NPY immunoreactive fibers in PVNmp may also contribute to the enhancement of hypothalamic CRH immunoreactivity and higher serum corticosterone observed in Npy1r^{rfb} mice.

In line with previous studies (34), we found that the increased CRH expression in the PVN of Npy1r^{rfb} mutants was associated with a lower density of CRH immunoreactive fibers in the CeA. This independent and opposite effect of *Npy1r* conditional inactivation on CRH immunoreactivity in the CeA and PVN may depend on the multiple feedback loops regulating the central CRH system in mature and developing rodents (35, 36). Thus, CRH at both the hypothalamic and the limbic sites may contribute to the behavioral, neuroendocrine, and metabolic phenotype associated with a decreased limbic Y1R expression.

One of the most significant findings of this study was the observation that differences in molecular, physiological, and behavioral phenotypes between Npy1r^{rfb} and Npy1r^{2lox} mice became apparent when both genotypes were raised by FVB/J, but not by C57BL/6J, dams. Quantitative analysis of maternal behavior in FVB/J and C57BL/6J foster mothers revealed remarkable strain variability. FVB/J dams showed a higher frequency of feeding and resting behavior and spent more time nursing their pups in the ABN position than C57BL/6J dams, whose foster litters showed decreased body weight from P6 onwards. These differences in maternal care during the first postnatal week of life might have long-lasting consequences for NPY/Y1R signaling. This hypothesis finds strong support by the increased levels of *Npy1r* mRNA in CA1, CA3, and DG of Npy1r^{2lox} mice raised by FVB/J dams compared with Npy1r^{2lox} mice raised by C57BL/6J foster mothers. As a possible implication, the FVB/J-fostered Npy1r^{2lox} control mice showed lower anxiety levels, higher body weight gain, and lower HPA activation than the C57BL/6J-fostered Npy1r^{2lox} controls.

In the brains of Npy1r^{2lox} and Npy1r^{rfb} mice fostered to C57BL/6J dams, the *Npy1r* mRNA levels were very similar and both mouse cohorts showed no significant phenotypic differences, suggesting that Cre-induced *Npy1r* gene inactivation cannot further decrease the overall *Npy1r* expression in excitatory forebrain neurons. The lack of conditional reduction of *Npy1r* gene expression in C57BL/6J reared mice cannot be attributed to alteration of transcriptional activity of the transgenic αCamKII promoter driving Cre, because the Cre immunoreactivity was comparable between Npy1r^{rfb} mice raised by FVB/J or C57BL/6J dams (Fig. S2).

The long-lasting impact of low levels of maternal care on offspring for anxiety and stress response in adulthood is well established (20, 37, 38). Low level of maternal ABN correlates with decreased hippocampal glucocorticoid receptor expression, decreased negative-feedback sensitivity, enhanced hypothalamic CRH expression, and higher HPA responses to stress in the adult. Given that NPY links excitatory stress response signals to activity of the HPA axis (25, 26, 39), an enhanced glucocorticoid negative feedback sensitivity may induce long-lasting effects on the cross-talk between the NPY and CRH systems, thus affecting anxiety and body weight gain. However, because maternal milk strongly influences the ability of the adrenal glands to secrete corticosterone in response to adrenocorticotropic hormone stimulation (40), we cannot exclude that milk composition or some other aspects of the maternal environment play a role in the development of the limbic NPY/Y1R system in pups under normal conditions.

In summary, we established a genetic tool to spatially and temporally reduce *Npy1r* expression in the forebrain of mice. Our analysis revealed that the conditional reduction of hippocampal Y1Rs increases anxiety-related behavior. In addition, we provide experimental genetic evidence that limbic Y1Rs are required for regulation of body weight. The reduced expression of the *Npy1r* gene in the forebrain apparently cannot be compensated for by adaptation to maintain homeostasis. Finally, our data indicate that neuronal NPY/Y1R pathways in the limbic system are key targets of maternal care-induced programming of anxiety and energy homeostasis.

Materials and Methods

Animals. Mice were caged in groups of 2–6, in a temperature- (22 ± 1 °C) and humidity- (50 ± 10%) controlled housing room on a 12-h light/dark cycle (0800–2000) 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/EEC) 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 Npy1r^{rfb} Mice. To generate the conditional deletion of *Npy1r*, a targeting vector for homologous recombination in ES cells was designed to introduce *loxP* sites around exons 2–3, which code for the entire region of *Npy1r*. The obtained *Npy1r* floxed mice were crossed with transgenic mice carrying a Dox-sensitive tTA-regulated Cre recombinase under the control of a limbic specific promoter (α-CaMKII). Using this combination of the tTA and Cre regulated gene expression systems, we achieved the deletion of *Npy1r* specifically in the α-CamKII positive excitatory neurons of the adult limbic system. Detailed information can be found in *SI Materials and Methods*.

Behavior. Behavioral tests were performed between P65 and P70 from 8 AM to noon (OF) or 5–7 PM (EPM). Locomotor activity in the home cage was continuously recorded with an infrared video camera, starting at the onset of the dark phase. Data were recorded automatically from the digitized image by using a computerized video tracking software. For maternal behavior analyses, pups were moved on P0 to Dox naïve foster mothers. Each dam was observed in her home cage for 2 h during the dark phase of the light/dark cycle, once every 4 min for a total of 30 observations on P1–P8. Detailed information can be found in *SI Materials and Methods*.

Histological Examination. Methods used for immunostaining, in situ hybridization, and quantification analysis can be found in *SI Materials and Methods*.

Serology. Methods used for serum collection and analysis (RIA and ELISA) can be found in *SI Materials and Methods*.

Data Analysis. Three-way ANOVA for repeated measures was used to compare mean body weight over time and food intake, and the appropriate contrasts were analyzed by unpaired *t* test. All of the other quantitative results were analyzed by two-way ANOVA, followed by Newman–Keuls test for multiple comparisons, or by the Student *t* test when indicated. All data are expressed as mean ± SEM, and the level of statistical significance was set at $P < 0.05$.

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