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"Effects of early maternal environment and limbic NPY-1r on behavior and reproduction of conditional KO female mice"

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Abstract (Italian)

Il fenotipo di un organismo, cioè l'insieme delle sue caratteristiche morfologiche e funzionali, è determinato dall'espressione del suo patrimonio genetico e dalle influenze dell'ambiente circostante. Nei mammiferi l'ambiente materno precoce riveste un ruolo fondamentale nel regolare lo sviluppo postnatale e il fenotipo. Studi condotti su roditori hanno dimostrato come la qualità delle cure materne influenzi comportamento, risposta allo stress e metabolismo della progenie in età adulta.

Nel modello murino caratterizzato dalla delezione condizionale (ristretta ai neuroni eccitatori del prosencefalo) del gene NPY-1r, che codifica per il recettore Y1 del neuropeptide Y (NPY), è stato dimostrato che gli effetti della delezione genica si manifestavano solo nei maschi allevati da madri adottive ad alto grado di cure materne; in questa condizione i topi knockout ($NPY1r^{rfb}$) mostrano minor peso corporeo, aumento del comportamento ansioso in età adulta, minori livelli plasmatici di leptina, minor quantità di tessuto adiposo e ridotta espressione dell'mRNA codificante per NPY-1r rispetto ai topi controllo ($NPY1r^{2lox}$).

Il presente studio ha lo scopo di esaminare il fenotipo comportamentale e metabolico di maschi e femmine knockout ($NPY1r^{rfb}$) e di controllo ($NPY1r^{2lox}$) allevati da madri caratterizzate da un diverso grado di cure materne e di indagare sul comportamento emozionale, riproduttivo e di motivazione materna delle femmine per valutare l'impatto dell'inattivazione condizionale di NPY-1r in area limbica e delle cure materne in risposta al challenge riproduttivo.

Dall'incrocio di tre diverse linee murine abbiamo ottenuto una prole costituita da topi $NPY1r^{rfb}$ e $NPY1r^{2lox}$ dati in adozione, alla nascita, a madri di quattro diversi ceppi caratterizzati da un diverso grado di cure materne, è stato osservato il comportamento materno spontaneo durante la prima settimana di vita dei piccoli, ed è stato monitorato il peso corporeo di maschi e femmine $NPY1r^{rfb}$ e $NPY1r^{2lox}$ dallo svezzamento all'età adulta.

Al raggiungimento dell'età adulta sono stati effettuati tre diversi esperimenti. Nel primo esperimento abbiamo indagato sugli effetti della delezione condizionale di NPY-1r sul comportamento emozionale (simil-ansioso, di risposta alla novità, e agonistico) e sul fenotipo metabolico in risposta a due differenti tipi di dieta in topi maschi e femmine $NPY1r^{rfb}$ e $NPY1r^{2lox}$ adottati alla nascita da madri di ceppo Balb/c, C57Bl/6J, CD-1 e FVB/J.

I risultati ottenuti dai test comportamentali hanno mostrato che in generale, i soggetti sperimentali erano caratterizzati da alti livelli di ansia ma la delezione condizionale di NPY-1r non sembra influire sul comportamento ansioso, mentre si osserva un effetto, dipendente dal genotipo e dal ceppo di adozione, sul comportamento aggressivo nei maschi: infatti i maschi

NPY1r^{rfb} allevati da madri ad alto grado di cure materne mostrano una riduzione del comportamento agonistico.

Il challenge metabolico costituito dalla somministrazione di una dieta ad alto contenuto calorico (HFD) non causa un aumento di peso nelle femmine, mentre nei maschi, indipendentemente dal genotipo e dall'adozione si osserva un aumento ponderale nel passaggio dalla dieta standard (STD) al regime di dieta ipercalorica. Questo effetto risulta avere una portata maggiore nei maschi NPY1r^{rfb} allevati da madri ad alto grado di cure materne, nei quali si osserva un incremento ponderale, un aumento dei livelli di glucosio plasmatici e un aumento del tessuto adiposo viscerale (WAT) rispetto ai maschi NPY1r^{2lox}.

Il secondo esperimento riguarda un challenge riproduttivo in femmine NPY1r^{rfb} e NPY1r^{2lox} accoppiate con maschi di ceppo C57Bl/6J e sottoposte a diversi test comportamentali, prima e durante la gravidanza: anedonia, esplorazione di un'arena, test di costruzione del nido, per valutare il comportamento simil-depressivo, ansioso e di motivazione materna. Al parto è stato registrato numero, sesso e peso dei piccoli e valutata la crescita e sopravvivenza delle nidiate. Le femmine NPY1r^{rfb} hanno avuto una significativa riduzione del successo riproduttivo (la percentuale di femmine che al PND 6 ha almeno un piccolo) rispetto ai controlli. Inoltre, i piccoli nati dalle femmine NPY1r^{rfb} mostrano alla nascita un peso corporeo minore rispetto ai piccoli delle femmine di controllo.

A differenza di quanto osservato nei maschi, in cui gli effetti della delezione genica di NPY-1r sono riscontrabili solo quando la prole è allevata da madri ad alto grado di cure materne, in questo studio è emerso che la delezione genica influenza la riproduzione nelle femmine e che questo effetto non sembra dipendere dalla qualità delle cure materne ricevute. Per comprendere se la riduzione del successo riproduttivo nelle femmine NPY1r^{rfb} sia attribuibile alla mancanza di motivazione materna o a una ridotta stimolazione da parte dei piccoli, nel terzo esperimento ho utilizzato la procedura di adozione incrociata tra femmine NPY1r^{rfb} e NPY1r^{2lox} che non ha fornito però dati conclusivi.

Sono tutt'ora in corso le analisi delle reti perineuronali, che potrebbero contribuire alla comprensione delle cause alla base della riduzione del successo riproduttivo osservato e del ruolo del sistema NPY limbico/recettore Y1 nella modulazione del comportamento riproduttivo.

Abstract (English)

The phenotype is the set of morphological and functional traits of an organism, resulting from the interaction between genotype and environmental factors. In mammals, early maternal environment plays a key role in the regulation of postnatal phenotypic development. Several studies in rodent models showed the influence of maternal cares on behavior, stress response and metabolism in adult offspring.

In the mouse model characterized by a conditional deletion of Y1 receptor of neuropeptide Y (NPY) gene in excitatory neurons of the forebrain, the gene deletion affected only male mice reared by high maternal care foster dams; knockout males ($NPY1r^{rfb}$) showed lower body weight, higher level of anxious behavior, lower plasmatic leptin levels, less abdominal adipose tissue and lower levels of NPY-1r mRNA expression compared to controls ($NPY1r^{2lox}$).

The aim of the present study was to investigate phenotypic differences in behavior and metabolism of $NPY1r^{2lox}$ and $NPY1r^{rfb}$ males and females, and if limbic inactivation of NPY-1r and/or early maternal environment affect emotional, reproductive and maternal behavior of females. The generation of $NPY1r^{rfb}$ and $NPY1r^{2lox}$ offspring was achieved by mating three different genetically modified mouse lines. At birth, litters were fostered to dams of four different strains characterized by different levels of maternal behavior, spontaneous maternal behavior was observed in the first week of pup's life and we monitor body weight from weaning to adulthood.

Upon reaching adulthood, three different experiments were performed. In the first experiment, we investigated the effects of conditional deletion of NPY-1r on emotional behaviour (anxious-like, novelty-response, and agonistic) and metabolic phenotype in response to two different types of diet in male and female $NPY1r^{rfb}$ and $NPY1r^{2lox}$ mice adopted at birth from mothers of Balb/c, C57Bl/6J, CD-1 and FVB/J strain.

The results obtained from the behavioural tests showed that in general, the experimental subjects were characterised by high levels of anxiety, but the conditional deletion of NPY-1r does not seem to affect anxious behaviour, whereas a genotype- and adoption strain-dependent effect on aggressive behaviour is observed in males: in fact, $NPY1r^{rfb}$ males reared by mothers with a high degree of maternal care show a reduction in agonistic behaviour.

When challenged with exposure to a high fat diet, female mice reduction of limbic NPY-1r or fostering at birth did not affect body weight growth on a standard diet and did not induce susceptibility to diet induced obesity. Conversely NPY1r^{rfb} male mice, especially when reared by high quality of maternal care foster mother were more susceptible to develop metabolic disorders. Although, regardless of the adoption, both NPY1r^{rfb} and NPY1r^{2lox} male mice fed HFD showed increased body weight growth than STD fed groups, NPY1r^{rfb} males showed much greater body weight gain, increased glucose blood levels, and higher amount of WAT than their control (NPY1r^{2lox}) when on HFD.

In the second experiment we performed a reproductive challenge in NPY1r^{rfb} and NPY1r^{2lox} females, mated with C57Bl/6J male mice. Before and during pregnancy females were subjected to different behavioural tests: sucrose preference test (anhedonia), open field and nest-building test were performed to evaluate depression-like, anxious and maternal motivation behavior. At delivery pups were sexed and weighed, and body weight growth and survival of offspring were monitored during the first postnatal week. NPY1r^{rfb} females showed decreased reproductive success compared to control females. Moreover, although no differences were observed in litter size, NPY1r^{rfb} females' litters showed lower body weight at birth compared to control females' litters. At difference with previous data on males, the effects of NPY-1r gene inactivation on female reproduction was affected by gene deletion regardless of the foster mother strains.

To assess whether the decreased reproductive success of NPY1r^{rfb} females was due to a lack in maternal motivation or to decreased pups stimulation we performed a third experiment which consists of a cross fostering experiment between NPY1r^{rfb} and NPY1r^{2lox} females. The data achieved by this experiment are not definitive. Currently analyses of of perineuronal nets are still in progress. These data studies can contribute to understand the mechanisms involved in the decreased reproductive success of NPY-1r^{rfb} females and the role of the limbic NPY system/Y1 receptor in the modulation of reproductive behavior.

Chapter 1

1.1 Neuropeptide Y (NPY)

Neuropeptide Y (NPY) is a mammalian 36 amminoacid peptide isolated for the first time in 1982 from pig brain by Tatemoto e colleagues (Tatemoto et al., 1982). NPY forms the family of pancreatic peptides, together with peptide YY (PYY) and the pancreatic polypeptide (PP) (Tatemoto et al., 1982). In rodents and humans NPY is widely distributed in central and peripheral CNS areas, such as: amygdala, hippocampus, nucleus of the solitary tract, locus coeruleus, nucleus accumbens and cerebral cortex (Allen et al., 1983; de Quidt & Emson, 1986; Dumont et al., 1992; Gustafson et al., 1986). NPY exerts several physiological functions, it is involved in neuronal excitability (Vezzani et al., 1999), neuroendocrine secretion (M. J. Morris & Pavia, 1998), regulation of food intake (Beck, 2000; Flood et al., 1987; Stanley & Leibowitz, 1984), metabolic functions (Krysiak et al., 1999; Small et al., 1997), circadian rhythm (White, 1993), cognition (Flood et al., 1987; Redrobe et al., 1999). NPY play a key role also in various psychiatric disorder, for example depression (Redrobe et al., 2002), anxiety (Ants Kask et al., 2002a) and epilepsy (Hökfelt et al., 1998). At peripheric level NPY is localized in neurons of the sympathetic nervous system (Lundberg et al., 1982): affects cardiovascular system and acts as an immunomodulatory element. It acts as a vasoconstrictor (Wahlestedt et al., 1990) and enhances the noradrenergic system's response to stress, furthermore, contributes to bone remodelling and lipid accrual (Shi & Baldock, 2012).

1.2 NPY receptors

NPY interacts with five different receptors that includes Y1, Y2, Y4, Y5 and y6 subtypes. They belong to a family of rhodopsin-like G-protein coupled receptors (Michel et al., 1998). NPY and PPY interacts with high affinity with Y1, Y2 and Y5 subtypes. Whereas PP shows selectivity to Y4 receptor binding (Bard et al., 1995). The subtype y6 (that exist as a truncated non-functional form in primates) is functional only in mouse and rabbit (Gregor et al., 1996). Several studies used different approach (ligand binding techniques, in situ hybridization, immunohistochemistry) to determine the localization of NPY receptors (Dumont et al., 1998; Herzog, 1999; Kopp et al., 2002). One of the most common pathways is the inhibition of the adenylate cyclase via pertussis toxin sensitive G-proteins (i.e. Gi and G0) that cause the decrease of cAMP cellular level (Michel, 1991; Olasmaa & Terenius, 1986). Another type of response is carried out via inositol

phosphate. IP induces the mobilization of intracellular Ca^{2+} stores that determine an increasing of intracellular Ca^{2+} concentration (Motulsky & Michel, 1988; Perney & Miller, 1989).

1.2.1 Y1 receptor

The first cloned (from rat forebrain) subtype among NPY receptors was Y1r, reported as an orphan receptor (Eva et al., 1990). Subsequently the human, mouse, monkey Y1 cDNA was isolated (Eva et al., 1992; Gehlert et al., 2001; Herzog et al., 1992; Larhammar et al., 1992). The Y1 receptor is a typical G protein-coupled receptor of the rhodopsin superfamily. The Y1 receptor exert his action through the inhibition of adenylate cyclase via pertussis toxin sensitive GTP binding protein and mobilization of Ca^{2+} from intracellular stores. Furthermore, Y1r regulates the mitogen-activated protein kinase (MAPK) pathway via phosphorylation of extracellularly regulated kinase (ERK) and this effect is PI-3-kinase dependent (Mannon & Mele, 2000). The Y1r gene is in the same cluster of Y5r on mouse chromosome 8B3-C2 and they are transcribed in opposite directions from a common promoter region suggesting that they have evolved from a gene duplication event (M. Nakamura et al., 1995). This mode of transcription of both genes from opposite strands of the same DNA sequence suggests that transcriptional activation of one of the receptors could modulate the gene expression of the other. Considering that Y1r and Y5r are involved in the regulation of food intake, it is likely that the coordinate expression of their specific genes can modulate the NPY activity. The expression of the Y1r seems to be under the control of different promoters activated in a tissue-specific manner (Ball et al., 1995). In most instance Y1r is located in post-synaptic sites with a distribution in several brain areas: cerebral cortex, hippocampus, mammillary nucleus, geniculate nucleus, limbic system (amygdala, bad nucleus of the stria terminalis) hypothalamus (medial preoptic area, paraventricular nucleus, dorsomedial, ventromedial and arcuate nuclei) (Kishi et al., 2005). The evaluation of physiological functions of the Y1 receptor relied for a long time on pharmacological tools but over the years several knockout murine models were developed (Pedrazzini, 2004). Despite the intermodal approach of pharmacological studies and the use of germline knockout models, data collected by pharmacological and in vivo studies has shown some conflicting results (S. Lin et al., 2004).

1.2.2 Y2 receptor

The Y2 is mainly a pre-synaptic receptor, implicated in inhibition of neurotransmitter release. The Y2r is expressed in SNC, intestine and blood vessel (Goumain et al., 1998; Rose et al.,

1995; Zukowska-Grojec et al., 1998). The Y2 receptor is accessible to circulating factors and mediating peripheral signals on the regulation of energy homeostasis (Broberger et al., 1997). In conditional Y2 receptor knockout mouse model the deletion of the receptor in the hypothalamus of these adult mice showed a significant decrease in bodyweight and a significant increase in food intake (Sainsbury et al., 2002). Furthermore, $Y2^{-/-}$ mice showed changes in behavioral traits such as anxiety-related behavior suggesting that the Y2 receptor has an inhibitory role on the anxiolytic-like effects of NPY (Redrobe et al., 2003; Tschenett et al., 2003).

1.2.3 Y4 receptor

The Y4 receptor is mainly expressed in the peripheric tissues such as the pancreas, intestine, colon, heart, and liver (Bard et al., 1995; Lundell et al., 1995). However, the expression of the Y4 mRNA and specific binding sites have also been found in the areas of the hypothalamus such as the paraventricular nucleus and in certain brainstem nuclei including the area postrema and the nucleus tractus solitarius (Herzog, 1999; Larsen & Kristensen, 2000). The Y4 receptor showed higher affinity for PP binding compared to the other NPY receptors.

1.2.4 Y5 receptor

The Y_5 receptor is distributed in several hypothalamic areas: Y_5 mRNA was detected in the medial preoptic area, paraventricular nucleus, suprachiasmatic nucleus, dorsomedial nucleus, ventromedial nucleus, lateral hypothalamic area, arcuate nucleus, and mammillary nuclei (Durkin et al., 2000; Larsen & Kristensen, 1998; Nichol et al., 1999). In the rodents, the distribution of Y_5R mRNA was rather similar to the distribution of Y_1R mRNA. The Y_5 receptor is involved in the regulation of anxiety along with Y_1 receptor.

1.2.5 y6 receptor

The y6 receptor for NPY is a pseudogene in humans and other primates (Matsumoto et al.,1996) and is missing in rat genome (Burkhoff et al., 1998), in pig and guinea pig is not functional (Starbäck et al., 2000; Wraith et al., 2000). The pharmacological characteristics of the y_6R were not clearly defined, and the roles of y_6R need further investigation.

1.3 NPY-1r and feeding

Among all the physiological functions of NPY, one of the most important is to stimulate feeding behavior, inducing, in particular, the intake of carbohydrates (Eva et al., 2006). Feeding behavior is essential in the regulation of energy homeostasis and it is closely regulated by neuronal, metabolic and endocrine signals integrated by the hypothalamus. Within the hypothalamus, NPY is mainly synthesized in neurons whose cell bodies lie in the arcuate nucleus (ARC) or dorsomedial hypothalamic nucleus (DMH) and send projections to adjacent areas, that are involved in the daily regulation of ingesting behavior and energy balance (e.g. paraventricular nucleus, ventromedial hypothalamic nuclei, and the lateral hypothalamic area) (Allen et al., 1983; Bai et al., 1985). There are also short projections in the ARC, populated by two subsets of neurons, orexigenic NPY neurons and anorexigenic proopiomelanocortin (POMC) neurons involved in the regulation of feeding control by NPY in an inhibitory manner (Marcos & Coveñas, 2021). It was demonstrated that the cerebral injection of NPY into either the ventricles or the hypothalamus, exerts a robust hyperphagia even in satiated rats, decreases energy expenditure, promote the lipogenic enzymes action in the liver and adipose tissues; overall these effects leads to to the development of obesity (Clark et al., 1984; Levine & Morley, 1984; Lin et al., 2006; Sainsbury et al., 1997). NPY is involved also in decreasing of thermogenesis, in brown adipose tissue (BAT), through inhibition of sympathetic outflow to BAT (Nakamura et al., 2017). Moreover, hypothalamic NPY synthesis and release in the ARC/PVN neurons are increased in poor metabolic conditions, such as starvation, lactation (Smith & Grove, 2002) or insulin deficient diabetes (Dube et al., 1992; Frankish et al., 1993). Leptin and insulin synthesis occur peripherally and through their releasing in the plasma, they act centrally to inhibit feeding and to increase energy expenditure (Könner et al., 2009). Among the orexigenic neuronal systems, NPY is a the most important neuropeptide implicated in mediating leptin action in the hypothalamus. Distinct neuronal populations in the ARC express leptin receptors and coexpress various neuropeptides including NPY (Dryden et al., 1995; Leshan et al., 2006; Stephens et al., 1995). Leptin acts an inhibitor of the synthesis and release of NPY and counteracts the effect of NPY on feeding, whereas NPY opposes the anorectic effect of leptin (Varela & Horvath, 2012). Similarly, central administration of insulin decreased the expression of NPY in rat ARC neurons whereas the NPY neurons become overactive when the levels of insuline fall during undernutrition (Dube et al., 1995; Schwartz et al., 1992; Sipols et al., 1995). Chronically elevated NPY-ergic tone has been shown to be associated with an obese status. Obese rodents fed with a 22-week high fat diet (HFD) (Huang et al., 2003) or gene mutation resulting in defective leptin signalling (Huijsduijnen et al., 1993; Mercer et al., 1996) show increased hypothalamic NPY

mRNA expression compared to lean controls. For most obesity cases, an elevated NPY-ergic tone is likely due to a central resistance to peripheral signals of energy excess such as leptin, which increases in response to a prolonged positive energy balance (Enriori et al., 2007; Fam et al., 2007; Huang et al., 2003; S. Lin et al., 2000). Among all the NPY receptors, Y1-r and Y5-r were involved in regulation of food intake (Wolak et al., 2003) and both are expressed in the hypothalamic sites implicate in the daily regulation of ingesting behavior and energy balance (Durkin et al., 2000; Kishi et al., 2005). The importance of the Y1r receptor subtype in regulation of feeding behavior was demonstrated also by pharmacological studies using selective agonists and antagonists. In rats, intracerebroventricular (icv) administration of NPY-1r agonists were shown to stimulate feeding and to induce hyperinsulinemia independently on food intake, (Mullins et al., 2001) whereas with used selective Y1-r antagonists, block appetite stimulation elicited by icv administration of NPY (Kanatani et al., 2001). Furthermore, peripheral administration of a highly selective Y1-r antagonist inhibits NPY-induced feeding and this compound is devoid of activity when administered to Y1-r knockout mice (Kanatani et al., 2001). After all, changes in feeding behavior and energy balance induce a marked plasticity in the Y1-r function and expression in specific regions of the hypothalamus, these evidence allows to underline the prominent role of Y1-r system in the stimulation of feeding and in the development of obesity (Eva et al., 2006).

1.4 NPY-1r in anxiety and stress

It is well known that one of the most important role of NPY consists of the regulation of emotional behavior and responsiveness to stressful stimuli. For example, intra-amygdalar or icv microinjection of NPY exerts a potent anxiolytic effect in several behavioral models of anxiety (Broqua et al., 1995; Karlsson et al., 2005; Pich et al., 1993; Sajdyk et al., 1999) and NPY knockout mice exhibit an anxiogenic-like phenotype (Bannon et al., 2000; Karl et al., 2008). Moreover, NPY inhibits several metabolic and behavioral responses to stress (Bannon et al., 2000; Broqua et al., 1995; Kask et al., 2002; Thorsell et al., 2000). The idea that a functional antagonism exists between NPY and the corticotropin-releasing factor (CRF) has been demonstrated in various nuclei involved in the stress/anxiety circuits such as the hippocampus (A. Thorsell et al., 2000), the hypothalamus (Hastings et al., 2001), the locus coeruleus (Charney, 2004), the periaqueductal grey (Charney, 2004) and the septal complex (Kask et al., 2001). NPY inhibits the anxiogenic-like effect of CRF (Charney, 2004; Kagamiishi et al., 2003; A. Kask et al., 2001), suggesting that this neuropeptide may act as a modulator to buffer against the stressor-induced release of CRF in the amygdala, a structure critical for the

generation, expression and maintenance of the emotional behaviors in both animals and humans (Davis, 1998; Reichmann & Holzer, 2016; Rogan & LeDoux, 1996). In addition, NPY has been shown to play as a neuromodulator agent on the hypothalamic-pituitary adrenal axis (HPA) (Heilig et al., 1994).

The anxiolytic action of NPY is primarily mediated through the NPY-1r system. Pharmacological studies demonstrated the involvement of the Y1 receptor in the anxiolytic effects of NPY using agonist, antagonist and antisense oligonucleotide of NPY-1r (Eva et al., 2006). NPY icv injections or directly into the central amygdala (CeA), elicits behavioral responses in several animal models of anxiety (Britton et al., 1997; Heilig, 1995; Sajdyk et al., 1999; Tasan et al., 2010). Conversely, using antisense oligonucleotide targeting to NPY-1r, were observed the opposite behavioral effects to the one induced by NPY administration (Heilig, 1995). Contrary to expectations, a restraint stress causes a decrease in anxiety in NPY-1r knock-out mice suggesting that compensatory changes in Y2r might be in part responsible for the altered phenotype of these animals (Thorsell et al., 1998; Wittmann et al., 2005). Several studies demonstrated that endogenous NPY signaling (that mediated mainly by Y1-r) is implicated in neuronal stress response.

The expression and content of NPY mRNA is dynamic and sensitive to acute and repeated restraint stress in several brain regions including forebrain, pons and medulla (Krukoff et al., 1999; Makino et al., 2000). However, it is not clear how the exposure to restraint may alter the brain expression of NPY, and the reasons why restraint induces increase, decrease and no changes in NPY mRNA and peptide depending upon strain, region or stressor paradigm. For example, exposure to acute restraint induce a reduction of NPY mRNA and protein concentrations in the amygdala and cortex of rats (Thorsell et al., 1998).

In contrast, chronic exposure to restraint, upregulates NPY gene expression in the amygdala and in the arcuate nucleus, suggesting that increased synthesis and release of NPY may act to ‘‘buffer’’ the behavioral effects of stress-promoting signals such as CRF (Annika Thorsell et al., 1999).

According with these observations, Mele et al. (2004) demonstrated that acute exposure to restraint increases Y1-r gene expression in the central and medial amygdala and PVN of Y1R/LacZ mice, suggesting that the up-regulation of the Y1-r may be a part of a compensatory mechanism triggered by the stress-induced reduction of functional NPY transmission in the amygdala. Conversely repeated restraint failed to affect Y1R /LacZ transgene expression in the amygdala and PVN of mice, suggesting that a change in Y1-r gene promoter transcriptional activity is not required for adaptation to restraint stress (Mele et al., 2004).

1.5 NPY-1r and reproduction

It is known that NPY, acting at all levels of the hypothalamic-pituitary-gonadal (HPG) axis, is a crucial neuronal modulator of several reproductive functions, including the generation of GnRH pulses and LH hormone surges, metabolic regulation of reproduction, puberty, and sexual behaviour. These effects depend on the environment of steroid hormones, NPY secretion levels, and the specific brain region. (Eva etal. 2006).

Furthermore NPY exerts a facilitatory role in the generation of preovulatory GnRH and gonadotropin surges (Crowley & Kalra, 1987; Kalra & Crowley, 1984) and this ability to elicit GnRH release and LH surge is increased immediately before proestrous (Besecke et al., 1994). Neuropeptide Y (NPY) has been reported to modulate the secretion of LH from the pituitary gland, possibly by affecting the secretion of LHRH from the hypothalamus and the sensitivity of the pituitary gland to LHRH (Minami et al., 1990). Furthermore, the LH surge is significantly decreased in NPY knockout mice (Xu et al., 2000). The effect of NPY on the GnRH occurred when progesterone and estradiol levels are high, during proestrous as NPY has a negative effect on GnRH levels, pulse amplitude and frequency when levels of estrogen and progesterone levels are low (Christian & Moenter, 2010; Hill et al., 2004; Khorram et al., 1987). Moreover, NPY acts as a mediator in communication among energy balance, GnRH secretion and sexual behaviour in many species (Ammar et al., 2000; Morris & Crews, 1990; Narnaware & Peter, 2001). Metabolic challenges, such as starvation and increased energy expenditure, could induces an increase in NPY production and release, and the inhibition of the pulsatile mode of LH secretion (Kalra et al., 1991; Lewis et al., 1993), and NPY levels are reduced by treatments that gain metabolic deficit and restore HPG axis function (Kalra et al., 1997).

The stimulatory and inhibitory effects of NPY on GnRH secretion are mainly mediated by Y1 receptors. Receptor Y1 has been implicated in the enhancement (Jain et al., 1999; Leupen et al., 1997) and the inhibition of LH release (El Majdoubi et al., 2000; Pralong et al., 2000; Sindelar et al., 2004) although also Y5R has been in part implicated in the inhibition of the LH surge (Raposinho et al., 1999). Y1R-positive fibers were found in close apposition to GnRH cell bodies in the preoptic area and Y1R mRNAs and NPYIR cells are also expressed in the anterior pituitary tissue and in gonadotrope-enriched pituitary cell cultures (Hill et al., 2004; Leupen et al., 1997).

Stimulatory effects of NPY through Y1-r on the LH preovulatory surge drawn from the observation that NPY and Y1-r agonists similarly stimulate LH release and that Y1-r selective antagonists attenuate both the LH surge in proestrous rats and surges induced by GnRH and

NPY in pentobarbital-blocked proestrous rats (Jain et al., 1999; Leupen et al., 1997; Besecke et al., 1994; Raposinho et al., 2000). NPY-induced augmentation of GnRH release occurs only during proestrous. It has been shown Y1-r mediated signalling may be influenced by steroid environment and that estrogens upregulate responsiveness to NPY through regulation of Y1-r gene expression in both the pituitary and the hypothalamus (Hill et al., 2004; Xu et al., 2000). Estrogens increase Y1-r gene expression through the regulation at transcriptional levels. 17β-estradiol increases Y1-r gene promoter transcriptional activity and this effect requires the presence of estrogen receptor-α and it is inhibited by co expression of estrogen receptor-β (Musso et al., 2000).

Inhibitory effects of NPY on LH secretion and sexual behavior were first proposed to be mediated by Y5-r since agonists that bind this receptor subtype inhibit LH release whereas Y5-r antagonists prevent NPY inhibition of LH surges (Raposinho et al., 1999). However, several evidence suggests that the Y1-r subtype also mediates NPY-induced inhibition of the gonadotrope axis. The central administration of a Y1-r antagonist to juvenile animals leads to the stimulation of LH release and to elicit precocious GnRH release (El Majdoubi et al., 2000; Pralong et al., 2000). Moreover, it has been suggested that the activation of Y1-r in the ARC inhibits sexual behavior through the activation of μ-opioid receptor in the medial preoptic area (Mills et al., 2004). This effect is inhibited by the selective Y1-r antagonist and is under the stimulatory or the inhibitory control of estrogen and progesterone, respectively.

Furthermore, studies performed on Y1-r knockout mouse model suggested a physiological role for the NPY-Y1r pathway in the adaptation of gonadotrope axis activity to metabolic changes. Adult Y1 receptor deficient mice show an increased resistance to the fasting-induced inhibition of the gonadotropin axis that is associated with increased circulating leptin levels (Pralong et al., 2000). The depletion of the Y1-r restores normal pituitary LH content and seminal vesicle weight in ob/ob mice, suggesting the existence of NPY effects independent on leptin action (Pralong et al., 2000). Furthermore, in juvenile mice lacking for the Y1 receptor, starvation fails to induce the expected delay of puberty despite the decrease of circulating leptin levels and the increase of NPY mRNA expression in the hypothalamus (Gonzales et al., 2004).

1.6 Generation of NPY1r^{rfb} conditional knockout mouse model

The mouse model used in this study, is a conditional knockout developed by Max Planck Institute of Heidelberg by Rolf Sprengel and described in detail in Bertocchi et al. (2011). The deletion of the NPY-1r gene is restricted to excitatory neurons of the forebrain. Lox P sites were inserted around exon 2 and exon 3 (which code for NPY-1r) using gene targeting procedure.

Inactivation of NPY-1r gene was achieved using recombinase Cre that promote site-specific recombination trough interaction with Lox P sites.

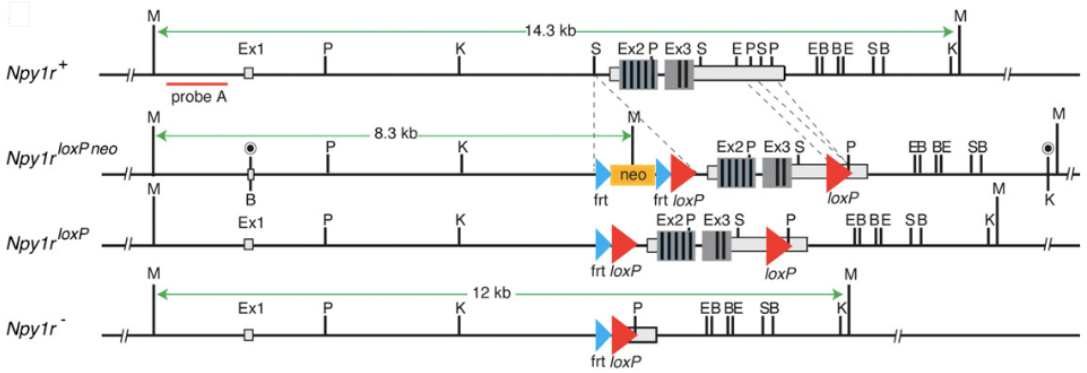


Fig. 1.1 Generation of a modified NPY-1r allele. To generate a *loxP* tagged *NPY1r* allele, *loxP* sites around exons 2−3, which cover the entire NPY-1r coding region, were inserted using standard gene targeting techniques in embryonic stem (ES) cells. Light grey boxes represent exons. Dark grey boxes are the coding regions with, in black, the trans-membrane domains. **B**: *BglII*, **E**: *EcoRV*, **K**: *KpnI*, **M**: *MscI*, **P**: *PstI*, **S**: *SphI*. Frt and *loxP* sited are in blue and red triangles, respectively. Circles indicate the **B** and **K** restriction sites; borders of the targeting vector. Arrows indicate the size of genomic fragments generated on Southern blotting by cleavage with *MscI.*

A mouse line carrying a floxed NPY-1r allele (NPY1r$^{+/loxP}$ mice) was generated by homologous recombination in embrionic stem cells (ES). Genomic DNA of the positive clones was digested with *MscI* and hybridized with a 1Kb probe, probe A, sitting outside at the 5" end of the targeting vector and obtained by PCR. In the Southern blot, wild-type allele was identified by the presence of a 14.3 Kb fragment, whereas recombinant allele by an additional 8.3 Kb fragment. ES cells of clone 441, positive for the recombination (NPY1r$^{+/loxP\text{-neo}}$), were expanded for injection into C57BL/6J blastocysts that were then implanted into pseudo-pregnant ICR female recipients. PCR and Southern blot analysis of tail DNA revealed that 50% of the pups contained the modified allele. To verify the efficiency of the Cre/*loxP* system, ubiquitous Cre-mediated deletion of the floxed exons was induced by using Cre-*deleter* transgenic mice, in which Cre is expressed in all tissues, including germ cells (Schwenk et al., 1995). Heterozygous mice NPY1r$^{+/-/CREdel}$, were analysedby PCR and Southern blot and then crossed to obtain the homozygous mouse. The ubiquitous Cre-mediated deletion of NPY1r was verified by *in situ* hybridization. The neo cassette was removed *in vivo* by crossing NPY1r$^{+/loxP\text{-neo}}$ mice to flp transgenic mice, carrying the Flippase (flp) recombinase, which recognizes and cuts at Flippase Recognition Target (frt) sites (Fig. 1.2 A). NPY1r mRNA expression was observed to extend along the rostral-caudal axis in the brain of NPY1r$^{+/loxP}$ mice and is reduced of about 50% in NPY1r$^{+/-}$ mice and completely absent in NPY1r$^{-/-}$ mice (Fig. 1.2 B).

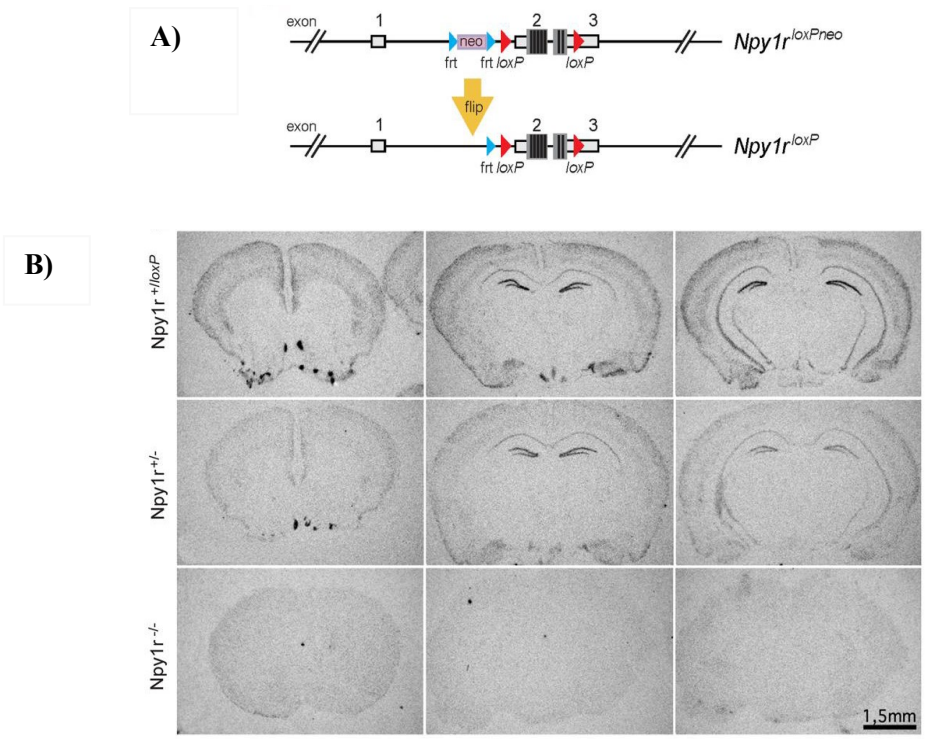


Fig.1.2 Verification of the efficiency of the Cre/*loxP* system. **(A)** The *neo* cassette was removed *in vivo* by crossing NPY1r+/*loxPneo* mice. **(B)** *in situ* hybridization on coronal brain sections from NPY1r$^{+/loxP}$, NPY1r$^{+/-}$ and NPY1r$^{-/-}$ germinal knockout mice confirmed the functionality of the LoxP sites at the targeted NPY1r^{loxP} locus.

Hereafter, in order to obtain the deletion of the NPY1r gene only in several brain regions, three different mouse lines were generated: 1. NPY1r^{2lox} expressing the LoxP sites flacking the NPY1r gene, 2. NPY1r^{2lox}/Tgα-CamKII-tTA mice line, expressing the tTA in a region- and cell type-specific manner under the control of the α-CamKII promoter (Mayford et al., 1996) and 3.

NPY1r^{2lox}/TgLC1 mice line, bearing a transgene containing the tTA-responsive promoter for Cre expression (Schönig et al., 2002). In this genetic paradigm, region-specific and postnatal inactivation of NPY-1r can be achieved by crossing NPY1r^{2lox}/Tgα-CamKII-tTA with NPY1r^{2lox}/TgLC1 mice and the extent of Cre expression could be restricted by Doxycycline (Dox) (Chiu et al., 2008; Krestel et al., 2004). In the resulting animals, chronic treatment with Dox from conception prevents NPY-1r inactivation by suppression of tTA-dependent Cre expression. Dox withdrawal, by switching litters at birth to Dox-free foster mothers, induces forebrain specific NPY-1r knockout (NPY1r^{rfb} mice) that is fully achieved around postnatal day P40. To assess temporal and spatial regulation of Cre activity *in vivo*, the pattern of Cre expression in the brain of Tg$^{α\text{-}CamKII\text{-}tTA/LC1}$ mice was assessed by means of immunohistochemistry. Cre immunostaining of coronal brain sections showed a specific, intense labeling of the Tg$^{α\text{-}CamKII\text{-}tTA/LC1}$ mouse anterior

forebrain, including the hippocampal formation, the cortex and the amygdaloid nuclei, indicating an α-CaMKII-driven Cre expression in these selected brain regions. Cre-like immunoreactivity was absent in the hypothalamus (Fig. 1.3 A). When Dox was applied from conception, consistently with previous results (Chiu et al., 2008; Krestel et al., 2004), no Cre activity was detectable in the brain. When Dox was applied from conception until birth and pups were shifted at PND0 to Dox-free foster mothers, the Cre specific immunosignal increased over time in the cortex, the hippocampus and the amygdaloid nuclei, reflecting postnatal increase in tTA from the transgenic α-CaMKII promoter (Chiu et al., 2008; Krestel et al., 2004) (Fig. 1.3 A). In order to increse the sensitivity of this analysis, $Tg^{\alpha\text{-CamKII-tTA/LC1}}$ mice were crossed to the RosaR26 Cre reporter mouse line, harboring a *lacZ* gene silenced with a floxed transcriptional terminator sequence (Soriano, 1999). Through an X-Gal staining, it was demonstrated that *lacZ* gene expression was similar to the regional distribution of Cre immunoreactivity (Fig. 1.3 B).

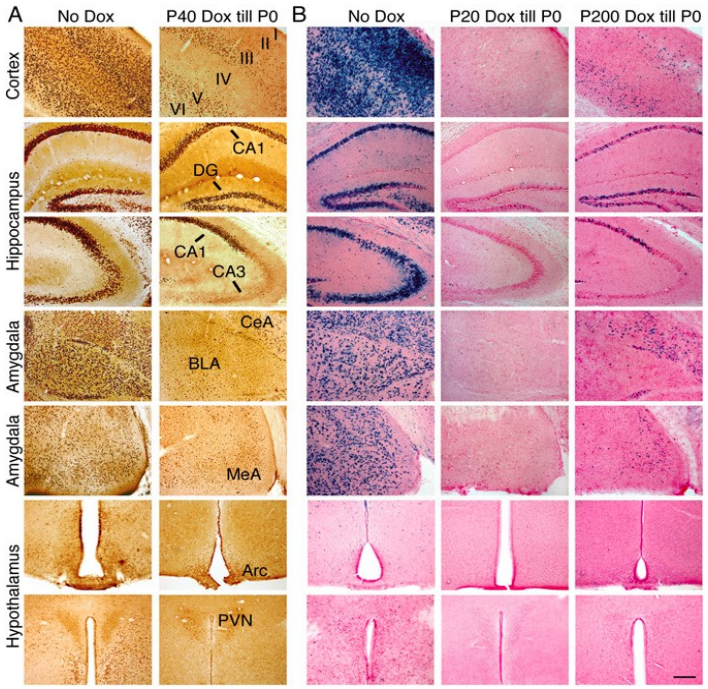


Figure 1.3. Temporal and spatial regulation of Cre activity. (A) Cre-immunolabeled and **(B)** corresponding X-Gal–stained (lacZ) coronal brain sections of Dox-naive (No Dox), and prenatally Dox-treated $Tg^{\alpha CaMKII\text{-}tTA/LC1}$ **(A)** and $Tg^{\alpha CaMKII\text{-}tTA/LC1/Rosa/R26R}$ **(B)** mice shifted at P0 to Dox-free (Dox till P0). All sections were taken at P20, P40, and P200. The lacZ gene of Rosa/R26R permitted the visualization of Cre activity by X-Gal staining. In Dox naïve mice, the Cre immunoreactivity of coronal brain sections showed a specific, intense labeling in the anterior forebrain of the $Tg^{\alpha CamKII\text{-}tTA/LC1}$ mouse, including the hippocampal formation, the cortex, and the amygdaloid nuclei. In contrast, anti-Cre staining was absent in the hypothalamus. When Dox was applied from conception until birth, the Cre-specific immunosignal was absent at P0 and increased over time in the cortex, the hippocampus, and the amygdaloid nuclei, reflecting postnatal increase in tTA from the transgenic αCaMKII promoter. Cre-induced lacZ expression was similar to the regional distribution of Cre immunoreactivity; Please note the absence of Cre-induced lacZ activity at P20. CA1, CA1 pyramidal cell layer; CA3, CA3 pyramidal cell layer; DG, dentate gyrus granule cell layer; BLA, basolateral amygdala; CeA, central amygdala; MeA, medial amygdala. (Scale bar: 200 μm)

1.7 Effects of of NPY1r^{rfb} conditional knockout

The effects of the conditional deletion of limbic NPY1r were first described by Bertocchi et al. (Bertocchi et al., 2011). In order to achieve the Cre recombinase activation and subsequently the conditional knock-out of Y1R, at birth (PND0) control (NPY1r^{2lox}) and conditional mutant (NPY1r^{rfb}) pups were fostered to two different mouse strains: C57Bl/6J and FVB/J.

Maternal behavior observation from PND1 to PND7 showed that FVB/J foster dams are characterized by higher level of maternal care (HABN) compared to C57Bl/6J dams (LABN). *In situ* hybridization of NPY-1r mRNA showed that the strain of foster mother influences the expression of limbic NPY-1r. In fact, in NPY1r^{rfb} mice fostered to FVB/J mothers, Cre recombination led to a significant reduction of NPY-1r mRNA expression in the hippocampal CA1 and CA3 pyramidal and in the dentate gyrus (DG) granule cell layers, compared with their control littermates. When litters were raised by C57Bl/6J mothers, NPY1r^{2lox} mice showed lower NPY-1r mRNA in CA1, CA3, and DG than FVB/J fostered NPY1r^{2lox} mice, suggesting that limbic NPY-1r expression depends on maternal care and that HABN might increase the expression of NPY-1r. More importantly, NPY1r^{rfb} mice fostered to C57Bl/6J mothers did not show the expected down-regulation of NPY-1r mRNA, possibly due to the already low NPY-1r expression in C57BL/6J-fostered NPY1r^{2lox} mice. These results suggest that limbic NPY-1r expression might be regulated through epigenetic mechanism by early maternal environment (Bertocchi et al., 2011) (Fig. 1.4 A-B). The differences in the expression of limbic NPY-1r reflect different behavioral and physiological consequences depending on the early maternal environment. In fact, only NPY1r^{rfb} male mice reared by FVB/J dams showed: lower body weight growth, lower serum leptin level, lower abdominal adipose tissue when compared to their control littermates (NPY1r^{2lox}). NPY1r^{rfb} mutants also displayed higher peripheral corticosterone and higher density of NPY immunoreactive fibers and corticotropin releasing hormone (CRH) immunoreactive cell bodies in the PVN suggesting that conditional inactivation of limbic Y1R might decrease body weight gain by activation of the HPA axis. On the contrary, no differences between NPY1r^{2lox} and NPY1r^{rfb} mice reared by C57Bl/6J were observed. NPY-1r conditional knock-out induced effects also on behavior. NPY1r^{2lox} and NPY1r^{rfb} mice were tested for anxiety-like behavior through Elevated Plus Maze and Open Field test. In both paradigms, NPY1r^{rfb} mice reared by FVB/J dams showed higher level of anxiety as compared to their control. No differences were observed in mice reared by C57Bl/6J. Interestingly, both the metabolic and the behavioral profile displayed by NPY1r^{2lox} and NPY1r^{rfb} mice reared by C57Bl/6J, was similar to those displayed by NPY1r^{rfb} reared by FVB/J. The conditional NPY-1r

KO mouse model thus provided the first experimental genetic evidence that limbic Y1-Rs are required for regulation of body weight and anxiety behavior and are key targets of maternal care-induced programming of energy homeostasis (Bertocchi et al., 2011).

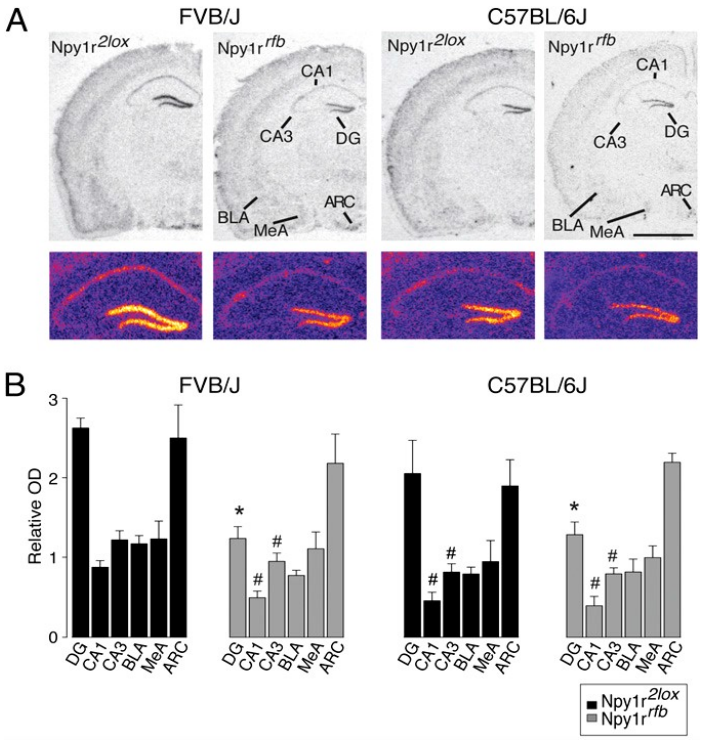


Fig 1.4 Expression of NPY-1r mRNA and Y1-r peptide in the brain of control and conditional mutants raised by FVB/J and C57Bl/6J dams. (A) Representative autoradiograms of in situ hybridization of NPY-1r mRNA on brain coronal sections of NPY1r^{2lox} and NPY1r^{rfb} mice fostered to FVB/J (Left) and to 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 NPY -1r 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 NPY-1r 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). CA1, CA1 stratum pyramidale; CA3, CA3 stratum piramidale; DG, dentate gyrus; BLA, basolateral amygdala; CeA, central amygdala; MeA, medial amygdala.

Chapter 2

2.1 Rational and purpose

In 2011, Bertocchi e colleagues, used the same conditional knockout mouse model described in this work. They demonstrated that the conditional inactivation of the NPY-1r gene exerts effects on males mice reared by HABN foster mothers on behavior, body weight growth, serum leptin levels, amount of adipose tissue and the expression of NPY and CRH hypothalamic expression.
In detail, $NPY1r^{rfb}$ male mice reared by FVB/J foster mothers, exhibit a reduced exploration and higher levels of freezing in the OF test and decreased amount of time and frequency of entries in open arms in EPM test; moreover they showed lower body weight, lower amount of adipose tissue and lower levels of leptin serum levels compared to $Npy1r^{2lox}$ males although no differences in food intake were recorded; furthermore, $NPY1r^{rfb}$ males displayed higher density of NPY immunoreactive fibers and CRH positive cell bodies in the PVN compared to their control littermates.
They showed that NPY/Y1R pathways in limbic area are modulated by early maternal care. Furthermore, following experiments showed that NPY-1r system display sexual dimorphic phenotype for behavioral and metabolic traits, and it is sensitive to gonadal steroids action (Bertocchi et al., 2020; Lin et al., 2004; Olofsson et al., 2009).
Previous experiments carried out in our laboratory in past years were focused to investigate the role of limbic expression of NPY-1r on behavior and metabolism of $NPY1r^{2lox}$ and $NPY1r^{rfb}$ mice of both sexes and fostered by dams with different levels of maternal care. The primary purpose of this work is to contribute to the knowledge of NPY-1r system and to investigate the effects of the conditional deletion of the Y1-r gene related to the early maternal environment on behavior and metabolism of $NPY1r^{2lox}$ and $NPY1r^{rfb}$ males and females reared at birth by different strain of foster mothers: Balb/c and C57Bl/6J that showed lower maternal care (LABN) conversely to CD-1 and FVB/J characterized by higher quality of maternal care toward pups (HABN).
The secondary objective was to assess if pregnant females could show phenotypic differences in reproductive behavior and maternal behavior due to the genetic background and the early maternal environment.
In the first experiment (Chapter 4) we investigated on emotional, agonistic, anxiety-like behavior and metabolism of $NPY1r^{2lox}$ and $NPY1r^{rfb}$ males and females fostered by HABN and LABN

dams of four different strains.

With the aim of understand if NPY-1r deletion and the early maternal environment could be affected reproduction and behavior during pregnancy in females, we exposed $NPY1r^{2lox}$ and $NPY1r^{rfb}$ females from the four different adoptions to a reproductive challenge described in second experiment (Chapter 5). In this experiment $NPY1r^{2lox}$ and $NPY1r^{rfb}$ dams were mated with C57Bl/6J wild type males, and we performed behavioral test to assess anxiety-like behavior, depressive-like behavior and maternal motivation behavior of dams. Moreover, we also recorded offspring features (number of pups for each litter, body weight of pups).

In the last experiment (Chapter 6) we exposed $NPY1r^{2lox}$ and $NPY1r^{rfb}$ fostered at birth by CD-1 foster dams to the same sequence of behavioral test described in Chapter 5, but at delivery we randomly assigned females to four groups (not cross fostered and cross fostered dams) as follows: 1) $NPY1r^{2lox}$ females left undisturbed with their pups; 2) $NPY1r^{rfb}$ females left undisturbed with their pups; 3) $NPY1r^{2lox}$ cross-fostered with $NPY1r^{rfb}$; 4) $NPY1r^{rfb}$ cross-fostered with $NPY1r^{2lox}$; at the end of behavioral test, $NPY1r^{2lox}$ and $NPY1r^{rfb}$ females were sacrificed and brains were collected to perform perineuronal nets analysis in the mPOA. Brain analyses are still in progress.

Chapter 3

3.1 General materials and methods

3.1.1 Animal and housing

All mice used in this work were born and reared in the Laboratory of Behavioural Biology at the University of Parma. Mice were housing with a temperature of (22 ± 1 °C) and humidity-controlled (50± 10 °C) room on a 12-hours light/dark cycle (11:00 AM – 11:00 PM). All the animals have *ad libitum* access to food (4RF21- standard diet, Mucedola, Milano) 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 Parma Ethical Committee for animal research and by the Italian Ministry of Health (Aut. N 1143-2016-PR Protocol 2712C.2 of 13/07/2016). The generation procedure of $NPY1r^{2lox}$ mice and $NPY1r^{rfb}$ was described in the next paragraph and in Bertocchi et al. (Bertocchi et al., 2011). In the meanwhile, female mice of four different strains BALB/c, C57Bl/6J, CD-1 swiss, and FVB/J used as foster mothers were paired with same-strain males. Females were kept with their respective males for 7-10 days. Pregnant females were initially housed in groups of two. Few days after birth mice were taken and placed in separated cages until delivery. Within 12 hours after birth (Post Natal Day 0) $NPY1r^{2lox}$ mice and $NPY1r^{rfb}$ pups were weighed, sexed, and fostered randomly to a lactating female belongs to one of the foster groups. Maternal behaviour was observed from PND 1 to PND 7, for two hours a day. At PND 27 pups were weaning and housed in groups of siblings of the same sex.

3.1.2 Generation of NPY-1r knockout mice and cross fostering procedure

For obteining $NPY1r^{2lox}$ and $NPY1r^{rfb}$ mice, two genetically modified mouse lines were crossed:

1) $NPY1r^{2lox/}Tg^{\alpha\text{-CamKII-tTA}}$ (expressing tTa under control of the promoter α-Ca^{2+} /calmoduline kinase II-tTa)
2) $NPY1r^{2lox/}Tg^{LC1}$ encoding LC1 (tTa responsive Cre transgene).

Breeding pairs were exposed to chronic Dox treatment from mating to the birth of pups (50mg/L in drinking water, 1% sucrose). At birth, littermates includes knockout mice (named $NPY1r^{rfb}$) and control mice (named $NPY1r^{2lox}$). At PND 0 mice were weighed, sexed, and fostered to Dox-free lactating females. Pups were weighed also at PND 10 and PND 27 (weaning). At weaning

all mice were marked with an ear tag and a PCR of tail DNA was carried out to verify the genotype. Mice were housed in groups (2-5 per cage) with same sex siblings.

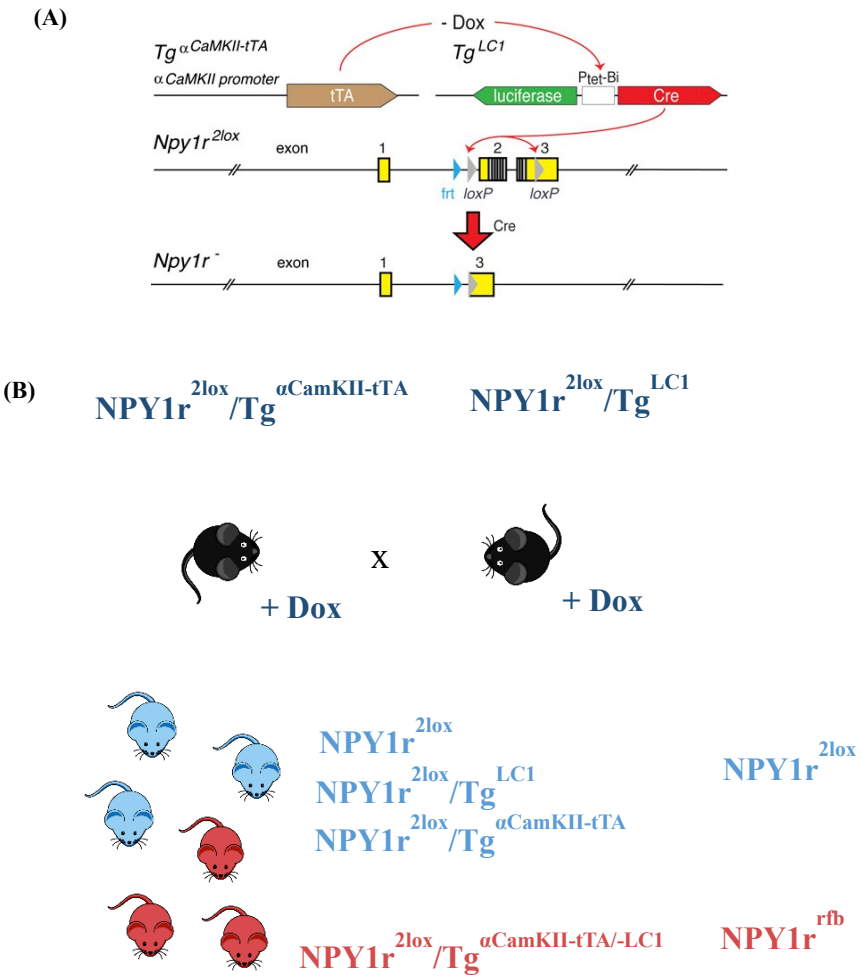


Fig. 3.1 Generation of NPY1r^{rfb} mutants and NPY1r^{2lox} control. (A) After Dox removal, the αCamKII promoter-driven tTA activates transcription of the transgene TgLC1, thereby inducing Cre expression in excitatory neurons of the forebrain. The Cre recombinase interacts with loxP sites in the gene-targeted NPY1r^{2lox} alleles and removes the NPY1r^{2lox} coding region leading to the inactivation of the NPY-1r 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. (C) At the day of birth, the litters were transferred to Dox naive foster mothers to induce the Cre-mediated NPY-1r 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).

3.1.3 Maternal behavior observation

Maternal behavior exhibited by foster mothers of 4 different strains was observed from PND 1 to PND 7. Foster dams was observed in their home cages in the last two hours of the dark phase of light/dark cycle (from 9:00 AM to 11:00 AM) because mice are most active during the dark

phase (Latham & Mason, 2004). Observations was conducted with the aid of red light lamp (25-W). At the end of two hours of test was collected a total of 30 observation for each lactating female, once every 4 minutes. Behavioral categories of maternal behavior was choosen according to Palanza et al. 2002 (Palanza et al., 2002), namely:

- **Arched back nursing** (the lactating female was arched with her body over pups, in order to give them nourishment and heath simultaneously)
- **Nursing** (the lactating female allows offspring to suckle)
- **Licking pup** (female was licking or grooming pups)
- **Nest building** (female was involved in nest building activity, inside or outside the nest)
- **Eating**
- **Drinking**
- **Grooming** (female was cleaning herself)
- **Active** (female was moving in the cage)
- **Resting** (female was outside the nest, not displaying any other behavior, motionless, without pups)
- **Out of nest** (female was anywhere in the cage, not interacting with pups)

Arched back nursing, nursing, licking pup and nest building were considered *pup-related* behavior, whereas others behavior described were considered *non pup-related* behavior.

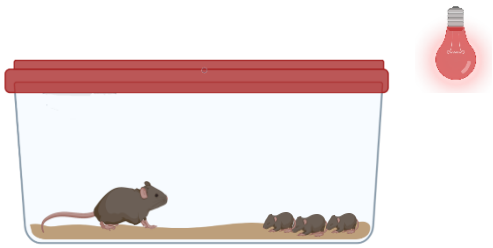


Fig. 3.2 *Maternal behavior observation scheme (*Created with BioRender.com)

3.1.4 Genotyping

Genotyping was performed on tail DNA at PND 27. All mice were identified by using the tTA gene as a marker gene for conditional knock-out. Animals positive for the tTA marker were considered as knock-out ($NPY1r^{rfb}$) whereas the others were controls ($NPY1r^{2lox}$). Procedure of DNA extraction was performed using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA). PCR was performed by using as a marker gene the tTA (primer:

tTA_1(GTGATTAACAGCGCATTAGAGC),tTA_4(GAAGGCTGGCTCTGCACCTTGGTG), Eurofins Genomics, Ebersberg) that was amplified in a 20ul reaction mix composed by: 1U of GoTaq Dna polymerase (Promega, Madison, WI, USA), dNTPS 0.2 mM, 8 pmol of each primer in 1X Reaction Buffer. The reaction was carried out at 95°C for 5 min (first denaturation step) followed by 34 cycles of 95°C for 40 sec, annealing at 56°C for 40 sec and elongation at 72°C for 1 min. At the end of the 34 cycles, the reaction was stopped through an elongation step at 72°C for 10 min. The presence of the PCR product (tTA) was evaluated through electrophoresis on 2.5% agarose gel.

3.1.5 Body weight growth monitoring (PND 34-PND 90)

Offspring body weight growth was monitored from PND 34 to PND 90 with a digital balance accurate to 0.01 g (Sartorius, Germany). The operation of weight was conducted with the animal weighing functions (animal's weight is automatically calculated as the average of a defined number of individual weighing operations). Animals were weighted once a week in the same range of time, from 10:00 AM to 11:00 AM.

3.1.6 Statistical analysis

A 2-way ANOVA (strain of foster mother and time) for repeated measures was used to analyse maternal behavior observation, while a 4-way ANOVA (sex, genotype, adoption, and PND) for repeated measures was run to analyse body weight growth from weaning to adulthood, A Tukey's test for post hoc comparisons followed the ANOVAs Data were analysed with Statistica 10.0 software (Stat-Soft, Tulsa, OK, USA). Significance was determined when $p<0.05$. Data are presented as mean ± standard error of the mean.

3.2 General results

3.2.1 Maternal behavior observation

The observation of spontaneous maternal behavior allows us to calculate the weekly percentage of time spent in pup related behavior and non-pup related behavior of foster dams of Balb/c, C57Bl/6J, CD-1 and FVB/J strains. As showed in picture, CD-1 and FVB/J foster mothers spent more time in arched back nursing (effect of dams strain: $F_{(3, 97)}=16,6256$; $p<0.001$) and nursing (effect of dams strain: $F_{(3,97)}=5,9613$; $p<0.001$) compared to Balb/c and C57Bl/6J dams; Arched

back nursing posture allows to provide heat and nourishment to the pups, a greater amount of time spent in arched back than in nursing posture suggest that CD-1 and FVB/J strains were characterized by higher levels of maternal care (HABN mothers) whereas Balb/c and C57Bl/6J showed lower levels of maternal care (LABN mothers). Furthermore CD-1 and FVB/J dams showed lower amount of time spent out of the nest (effect of dams strain: $F_{(3.97)}$=16,9539; p<0.001) and in others activities (effect of dams strain: $F_{(3,97)}$=33,6636; p<0.001) compared to Balb/c and C57Bl/6J.

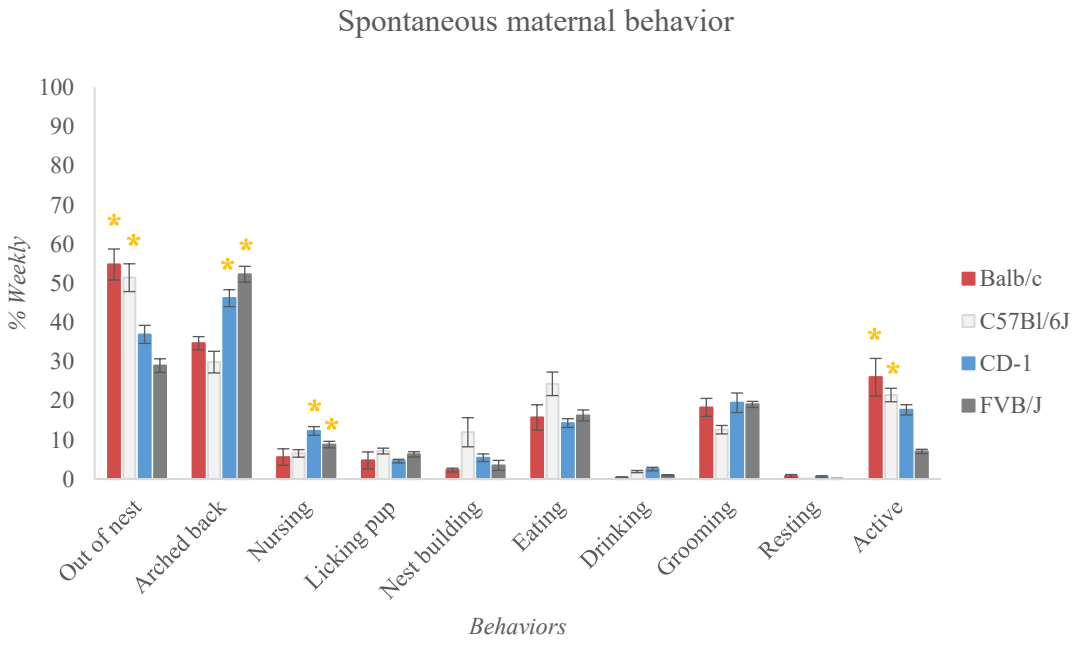


Fig. 3.3 Maternal behavior of Balb/c, C57Bl/6J, CD-1 and FVB/J foster dams. Average percentage of time spent in spontaneous maternal behavior of Balb/c, C57Bl/6J, CD-1 and FVB/J dams in the first week of pups life (PND1-PND7). Data are presented as mean ± SEM, *indicates a significative difference with p <0.05.

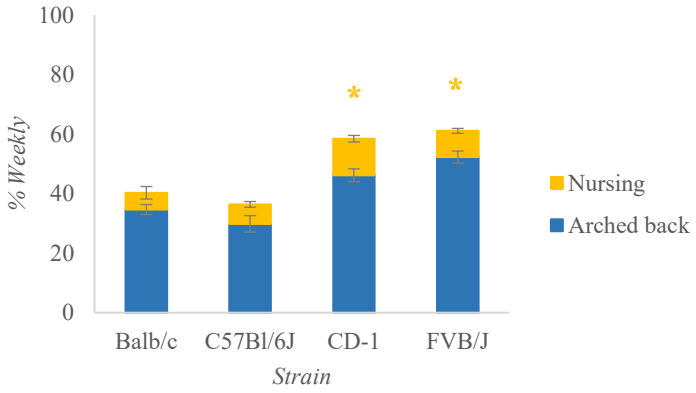


Fig. 3.4 Percentage of total nursing (Arched back and Nursing) of Balb/c, C57Bl/6J, CD-1 and FVB/J foster dams. Average percentage of time spent in total nursing behavior of Balb/c, C57Bl/6J, CD-1 and FVB/J dams in the first week of pups life (PND 1-PND 7). Data are presented as mean ± SEM, *indicates a significative difference with p <0.05.

3.2.2 Monitoring of body weight growth (PND 34-90)

From PND 34 animals were weighted weekly until PND 90. Overall, regardless of genotype, there is a difference between males and females (main effect of the sex: $F_{(1,217)}$ =134.25; p < 0.001), but also the strain of the dams seems to act on the statistical analysis (effect of fostering: $F_{(3,217)}$ =31.93; p< 0.001). Body weight increases in males and females mice during days in both genotypes (interaction between PND, sex and genotype: $F_{(8,1736)}$=2.95; p=0.002804), with an interaction among sex and fostering ($F_{(24,1736)}$ =2.76; p< 0.001). Thereafter we analysed separately males and females. In males there is an effect of genotype and adoption (respectively $F_{(1,111)}$=5.62; p=0,019449 and $F_{(3,111)}$=23.04; p<0.001), in the interaction between days and genotype ($F_{(8,888)}$=4.72; p=0,000011) and in the interaction between days and adoption ($F_{(24,888)}$=53.29; p<0.001). In females results showed a significative effect of adoption ($F_{(3,106)}$=10.62; p=0,00004) and of the interaction between days and adoption ($F_{(24,848)}$=28.10; <0.001).

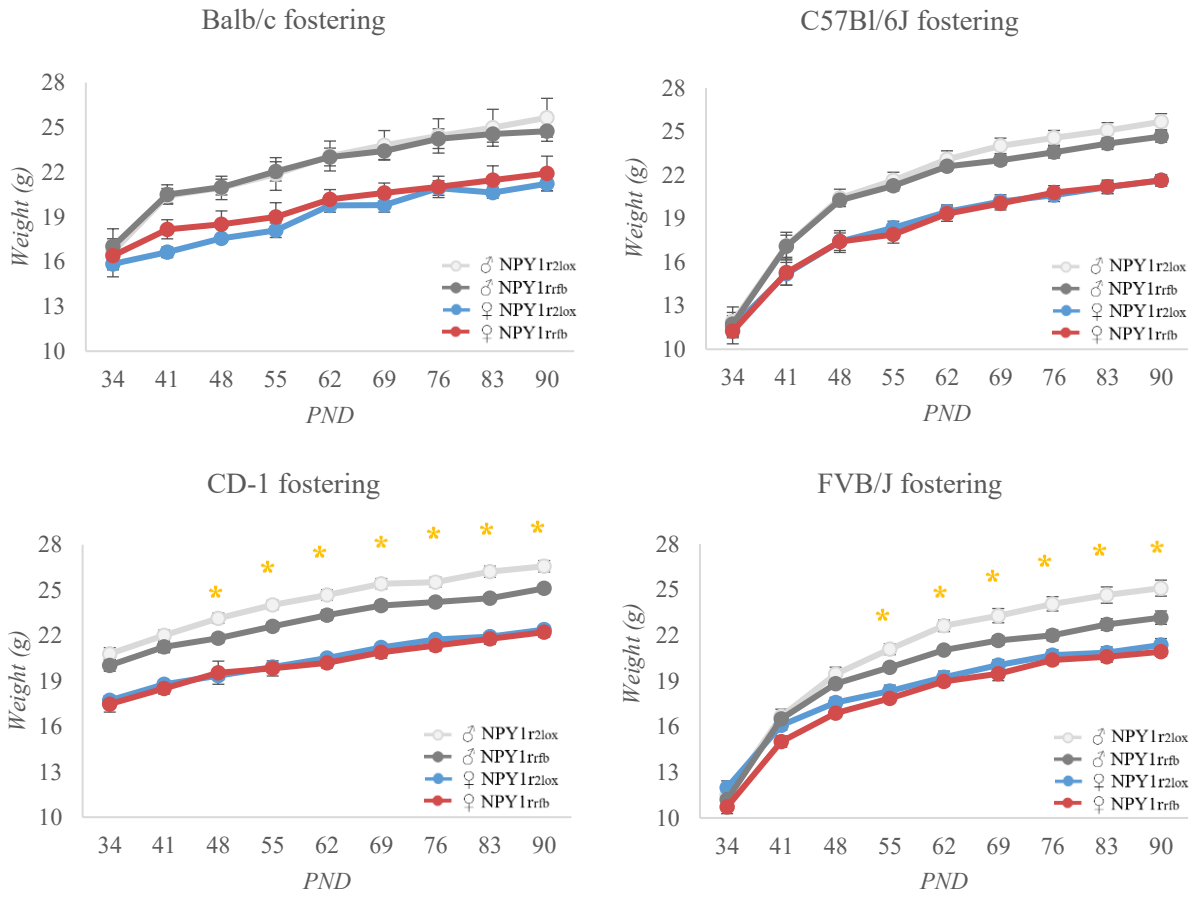


Fig. 3.5 Body weight growth of NPY1r^{2lox} and NPY1r^{rfb} male mice reared by, Balb/c, C57Bl/6J, CD-1 and FVB/J foster dams. Starting from PND 48, NPY1r^{rfb} male mice reared by FVB/J and CD-1 showed a lower growth rate as compared to NPY1r^{2lox} littermates. *indicates a significant difference between NPY1r^{2lox} and NPY1r^{rfb} with p<0.05.

3.3 Discussion

The first step of this work was the generation of the conditional knockout model used for the experiments described in following sections. By the crossing of three different murine lines we obtained NPY1r^{2lox} and NPY1r^{rfb} males and females offspring, fostered, at birth by four different strains of dams. In the first week of pups life we assess spontaneous maternal behavior. Our data showed that CD-1 and FVB/J spent a greater amount of time in nursing behavior, both arched back nursing posture and nursing posture, compared to Balb/c and C57Bl/6J dams. Data suggest that CD-1 and FVB/J dams were characterized by higher levels of maternal care (HABN) opposite to Balb/c and C57Bl/6J (LABN) that showed lower levels of maternal care. Furthermore, conversely to HABN dams, LABN dams spent a lot of time out of nest and in other non-pups related behavior. Statistical analysis revealed that HABN mothers showed significative higher amount of time spent on total nursing (arched back and nursing posture) and lower levels of time spent in other activities, out of the nest, compared to LABN dams. Starting from weaning to adulthood (PND34 - PND90) we carried out monitoring of bodyweight of NPY1r^{2lox} and NPY1r^{rfb} males and females from the four adoptions.

As we expected, females showed lower bodyweight compared to males, regardless of the strain of foster mothers. Only when reared by CD-1 and FVB/J dams, NPY1r^{rfb} males showed lower bodyweight compared to NPY1r^{2lox} littermates. Taken together these data confirm what reporting by Bertocchi and colleagues, the involvement of NPY-1r system in the regulation of metabolism and the early maternal environment can exerts an effect in programming the effects of limbic NPY-1r in energy homeostasis. Furthermore, the effects of the conditional deletion of NPY-1r are different between sexes. In following chapters, we investigate sex-differences in metabolism and behavior of NPY1r^{2lox} and NPY1r^{rfb} mice, with a focus on reproductive in females.

Chapter 4

4.1 Experiment 1: Effects of early maternal environment and limbic NPY-1r expression on behavior and metabolism in NPY1r^{2lox} and NPY1r^{rfb} males and females reared by different strain of foster mothers

As described in the previous chapter, the first step of the experiment was the generation of NPY1r^{2lox} and NPY1r^{rfb} offspring, reared at birth, by four different strains of foster mothers. In the first week of pup's life maternal behavior was observed, and body weight of pups were collected from PND 34 to PND 90. In adulthood NPY1r^{2lox} and NPY1r^{rfb} males and females fostered by HABN and LABN dams were tested for behavioral and metabolic challenge. To assess if conditional deletion of NPY-1r gene and early maternal environment can affect anxiety like, agonistic and aggressive behavior, males and females were subjected to elevated plus maze test, novelty induced suppression of feeding test and resident-intruder test. Furthermore, with the aim to rate metabolic alterations due to reduced limbic NPY-1r expression, NPY1r^{2lox} and NPY1r^{rfb} mice were exposed to two different diets (a standard diet and an hypercaloric diet) and glucose blood levels were detected through glucose tolerance test. Behavioral tests were carried out between PND 90 and PND 110 from 3 PM to 7 PM. At PND 90 animals were isolated in plexiglas cages (45cm*25cm*20cm) and, after a habituation period of 1 week.

4.2 Materials and methods

4.2.1 Elevated Plus Maze test (EPM)

NPY1r^{2lox} mice and NPY1r^{rfb} reared by HABN / LABN dams were tested in the EPM in a half-lit room to simulate the dark phase that is known to be the more active phase of rodents. The EPM apparatus consists of a plus-cross shaped (30*5cm) originating from a central platform (5*5cm) elevated 40 cm above the floor. Two arms, opposing each other, are enclosed by plexiglas walls (20 cm) and the leftover arms are open. All mice were transported to the testing room at least 1 hour prior the behavioral test for habituation. Each animal was placed on the center platform facing one of the open arms and was allowed to explore the maze for 5 minutes. The test was recorded with a video camera placed above the apparatus. To reduce any persistent olfactory cues, the maze was cleaned with clean damp cloth and a solution of 2% ethylic alcohol between successive trials. At the end of the procedure, we analysed the amount of time spent in

each zone of the maze and the frequency of transitions between different arms by an observer, previously trained, using specific software (The Observer, Noldus, NL).

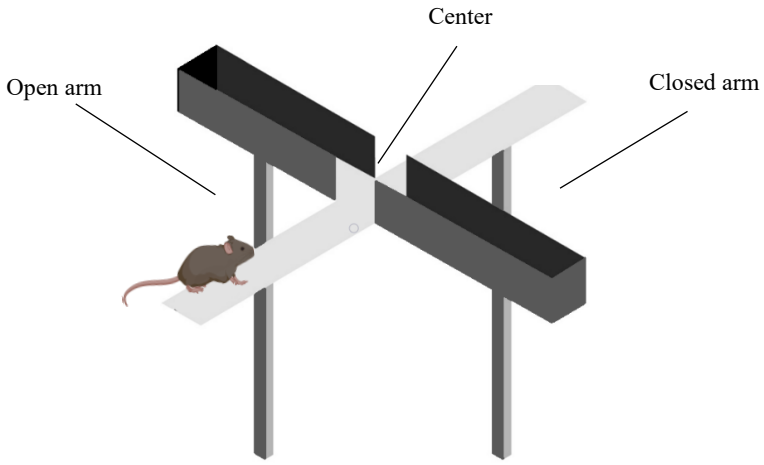


Fig. 4.1 *Elevated plus maze test (EPM) scheme (Created with BioRender.com)*

4.2.2 Novelty Induced Suppression of Feeding test (NISF)

To measure the latency of an animal to approach and eat a familiar food in a novel environment and assess the animal anxiety-like state NISF test is very effective (Dadomo et al., 2011). The paradigm of NISF test consists of to place a little piece of peanut (a palatable food for mice) in a Petri dish located in a corner of their home cage and latency of animals to eat the peanuts was collected. We repeat this procedure for three consecutive days for each experimental subject. On the fourth day, the Petri dish containing the peanut was presented in a novel cage with fresh bedding (Merali et al., 2003). The latency was recorded on all days with a cut-off time of 600s. In case of maximum latency, the peanut was left in the cage of the animal. A reduced latency to begin consumption of the palatable snack in the novel cage indicate a reduction of the anxiety, using this test.

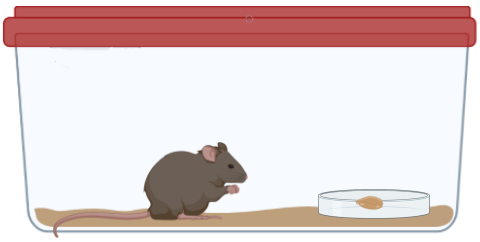


Fig. 4.2 *Novelty induced suppression of feeding (NISF) test scheme (Created with BioRender.com)*

4.2.3 Resident/Intruder test

Aggressive and social behavior was assessed with Resident/Intruder paradigm. The animals were individually housed and in the homecage of each experimental subject (the resident), were placed a same-sex intruder of BALB/c strain, which are known to show low level of aggression. Experimental animals were observed and video-recorded for 10 minutes with a camera. Latency to the first attack by the resident against the intruder was recorded and the test was terminated if clear signs of distress in the intruder transpired. Behavior was scored by the software The Observer (Noldus, NL). Specific behaviors analysed were: numbers and duration of attack, tail rattling (rapid vibration of the tail, typically occurs shortly before and during an attack flurry), circling and chasing (the resident chases rapidly the intruder shortly before an attack occurrs), upright posture (following being defeated, mice rear up on their hindlegs and assume a defensive upright posture), immobility with contact (resident mice are in a freezing posture with contact to the intruder), startle response (defensive response to threatening stimuli), social exploration (resident explores the intruder, it includes ano-genital exploration), immobility without contact (resident mice are in freezing posture within the cage), rearing (mice rear up on their hindlegs), digging (mice dig bedding), environment exploration and walking. In order to promote a clear representation of the data, specific behavioral elements were grouped into the following broad behavioral categories: offensive behavior (attack, tail rattling, circling and chasing) defensive behavior (upright posture, immobility with contact and startle response) and social behavior (exploration of the intruder, social interaction).

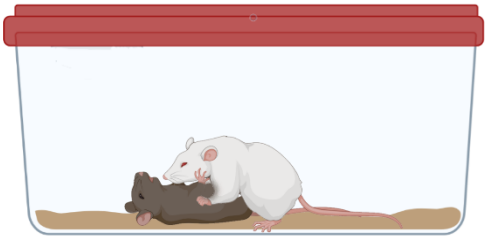


Fig. 4.3 *Resident-intruder test scheme (Created with BioRender.com)*

4.2.4 Body Weight and Food Intake

On PND 120 male and female NPY1r^{2lox} and NPY1r^{rfb} mice were weighed and individually housed in plexiglass cages (45cm*25cm*20cm) with wood shaving bedding changed weekly. After the first week on a standard diet, to obtain baseline measurement, NPY1r^{2lox} and NPY1r^{rfb}

reared by HABN and LABN mothers were randomly assigned to two experimental groups: one fed standard diet (STD - 6.55% kcal from fat and 3.9 kcal/g; 4RF21, Mucedola, Italy) and the other fed a custom pelleted high fat diet (HFD - 45% kcal from fat and 5.2 kcal/g manufactured by Mucedola) obtained by a modification of the STD formula. Throughout the study, food and water were available ad libitum to all experimental animals. Body weight and food intake were determined from 10 AM to 11 AM every two days for 3 weeks. Pre-weighed food was carefully placed in the U-shaped metallic feed hopper of each cage and then reweighed on a per-cage basis. Food crumbles with a diameter greater than 1 mm were recovered from the cage and weighed as well. Food intake was quantified by calculating the difference between pre- and post-weighed food for the entire duration of the experiment and averaged over baseline and weekly. Food intake was transformed to kcal and expressed as fold changes vs baseline.

Fig. 4.4 *STD diet and HFD diet (Created with BioRender.com)*

4.2.5 Glucose Tolerance test (GTT)

On the 21st day of the diet, Glucose Tolerance Test was performed following overnight fasting (12h). Blood glucose levels obtained from tail bleeding were monitored at 0, 30, 60 and 120 min after i.p. injection of d-glucose (Carlo Erba Reagent S.r.L., Milan, Italy) at 1g/Kg. Blood concentrations were all determined through an Accucheck Aviva (Roche Diagnostics, USA) blood glucose meter during the light phase between 9:00 AM and 11:00 AM. Mice were then returned to their diet regimen for 5 days to allow body weight recovery.

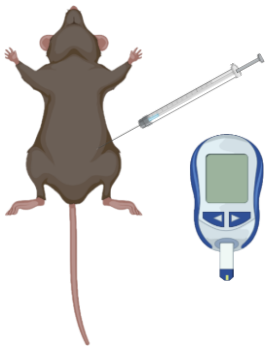


Fig. 4.5 *Glucose tolerance test (GTT) scheme (Created with BioRender.com)*

4.2.6 Tissue collection and plasma analysis

On day 26 of diet exposure, following 12 hours fasting, mice were euthanized by decapitation after a brief CO_2 exposure. Trunk blood was collected in heparinized tubes (Sarsted, Germany) and centrifuged at 4000 RPM, at 4°C for 10 min, then placed at -20°C until analysis. Abdominal white adipose tissue (perigonadal, visceral, retroperitoneal fat pads) was collected and weighed, then frozen in liquid nitrogen for later analysis. Brains were removed, immediately frozen in liquid nitrogen, then stored at -80°C until analysis.

4.2.7 Statistical analysis

A 3-way ANOVA (genotype, adoption and time) for repeated measures was used to analyse body weight, food intake and glucose tolerance test data. A 2-way ANOVA (adoption and genotype) was run to analyse abdominal fat pads, and behavioural tests. A Tukey's test for post hoc comparisons followed the ANOVAs.

4.3 Results

4.3.1 Elevated plus maze (EPM) test

Data were analysed using parametric analysis of variance (ANOVA) with strain of foster mother (Balb/c, C57Bl/6J, CD-1 and FVB/J), genotype ($NPY1r^{2lox}$ vs $NPY1r^{rfb}$) and gender (males and females) as between-subjects factors, and total duration, total frequency and zones of the EPM as within-subjects repeated measures. All the animals were analysed for total frequency of entrance and total time spent in the three zones of EPM (closed arms, open arms, center). Regardless of sex, all subjects spent more time in the closed arms of the apparatus. We observed a main effect of the genotype in total time spent in the closed arms of the maze ($F_{(1,240)}$ =5.3008; p=0.022172), with $NPY1r^{2lox}$ animals that often spent more time in closed arms when compared with their respective $NPY1r^{rfb}$. Moreover, this effect seemed to be connected also to sex and adoption: in fact, $NPY1r^{2lox}$ males reared by C57Bl/6J, Balb/c and FVB/J spent more time in closed arms than their respective $NPY1r^{rfb}$. About females, $NPY1r^{2lox}$ reared by C57Bl/6J spent less time than their $NPY1r^{rfb}$ in closed arms of the maze, whereas females $NPY1r^{2lox}$ reared by Balb/c, FVB/J

and CD-1 dams showed an increase in time spent in closed space when compared with their NPY1r^{rfb} (interaction between sex, genotype and adoption: $F_{(3,240)}$ =2.7456; p=0.043683). Regard total time spent in the center zone of the maze, overall NPY1r^{2lox} animals spent less time than NPY1r^{rfb} mice exploring the center (main effect of genotype: $F_{(1,240)}$ =14.9934; p<0.001). We only observed a direct effect of the fostering when we analysed total time spent in the open arms of the apparatus ($F_{(3,240)}$ =5.26513; p=0.001556), with mice reared by Balb/c and FVB/J showing less time spent in the open arms of the maze than the other groups.

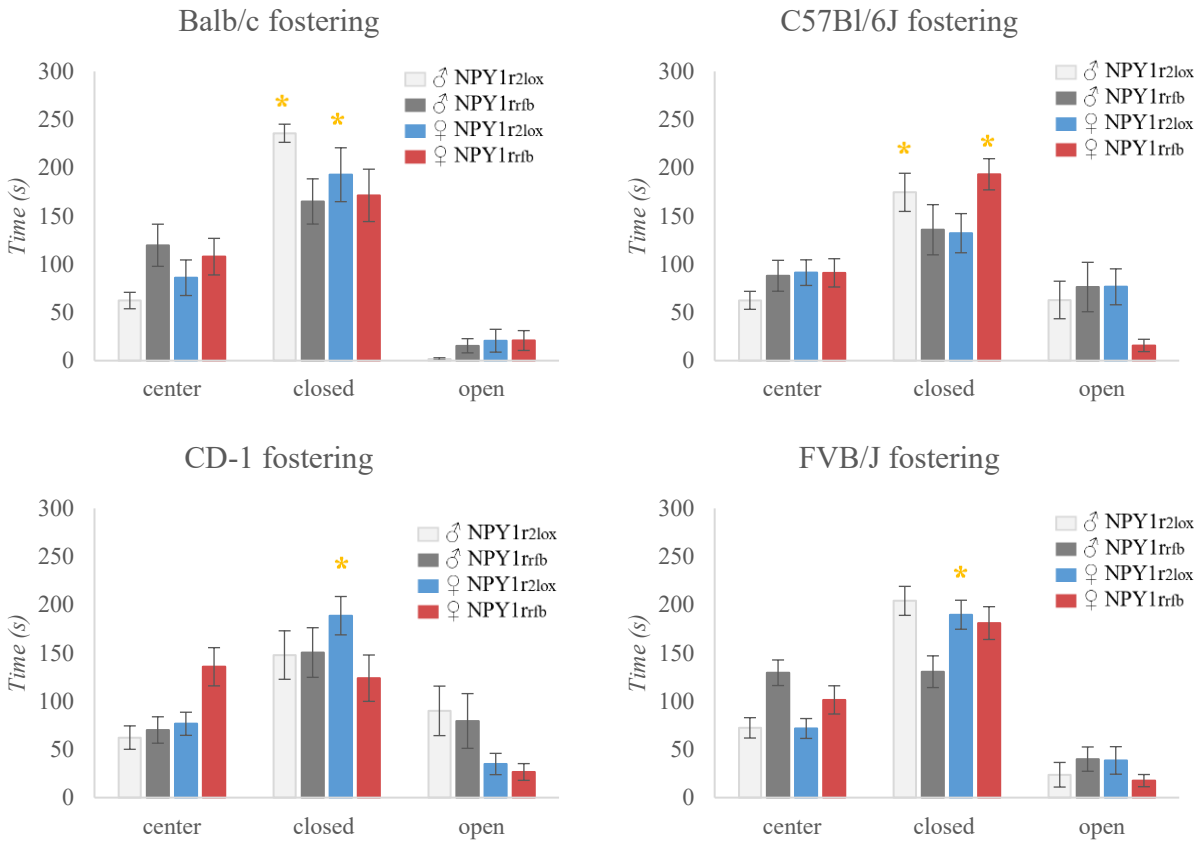


Fig. 4.6 Elevated Plus Maze test. Time spent in the center, open and close arm of EPM. Interaction among sex, genotype and adoption was observed in total duration spent in closed arms of the maze. Main effect of genotype detected in time spent in the center of the apparatus; effect of the adoption detected in time spent in open arms. Values are presented as mean ± SEM.

When we analysed the frequency of entrance in the three zones of the EPM it emerged that the number of transitions in the closed arms of the maze was higher than in the open arms, according with the results of the total duration previously showed. Overall, regardless sex, there is an effect of genotype ($F_{(1,240)}$=23.1100; p<0.001) and fostering ($F_{(1,240)}$ =4.0140; p=0.008199) on the closed frequency: NPY1r^{2lox} entered more times in the closed arms of the apparatus than NPY1r^{rfb} mice, and mice reared by C57Bl/6J and FVB/J dams, regardless of the genotype, presented less frequency of entrance in the closed space of the maze. About the frequency in the

open arms, males reared by Balb/c, and marginally, by FVB/J mothers showed less frequency of entrance in the open arms than the other groups (interaction between sex and fostering: $F_{(1,240)}$ =3.2949; p=0.021233).

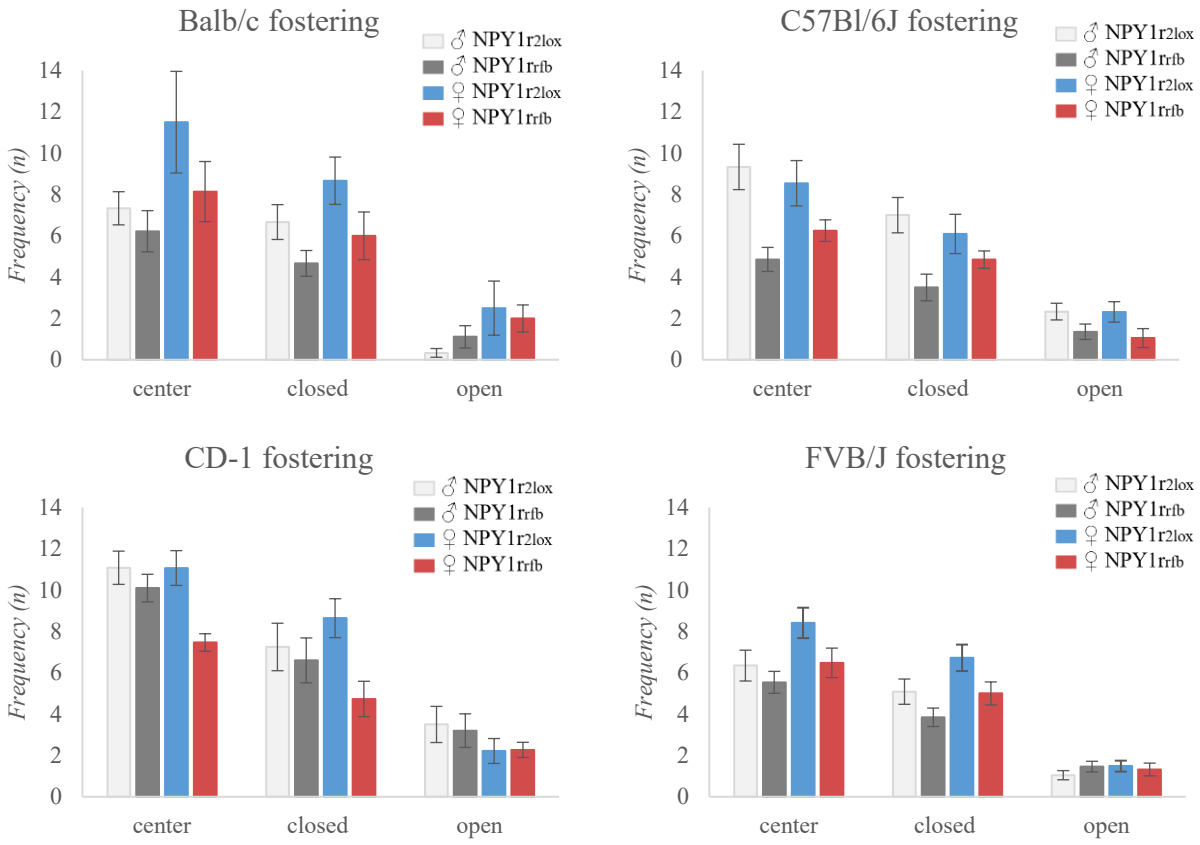


Fig.4.7 Elevated plus maze test. Frequency of entries in open and close arms and center of EPM. No significant differences were observed between males and females in total numbers of entrance in closed and center. Main differences were due to genotype and adoption. Values are presented as mean ± SEM.

4.3.2 Novelty induced suppression of feeding (NISF) test

NISF test provide the results on latency to consumption of a palatable snack over habituation and during the test in a novel environment. NPY1r^{rfb} and NPY1r^{2lox} mice reared by HABN or LABN mothers have been tested for NISF for 4 days, 3 days of habituation in their homecage and 1 day of test in a new cage. Regardless of gender, there is a significant decrease in latency to eat the palatable food during the habituation (main effect of the days: $F_{(2,510)}$ =173.7857; p<0.001), but no effect of fostering, genotype or sex was detected. During the test (day 4) we observed a main effect of the genotype ($F_{(1,255)}$=9.2848; p=0.002553) and a tendency of the interaction between sex and genotype ($F_{(2,510)}$=3.5640; p=0.060182). In fact, when individually analysed, regardless the fostering strain males showed an effect of the genotype: NPY1r^{2lox} presented a higher latency

when compared with NPY1r^{rfb} male mice. No significant differences were detected in females at day 4.

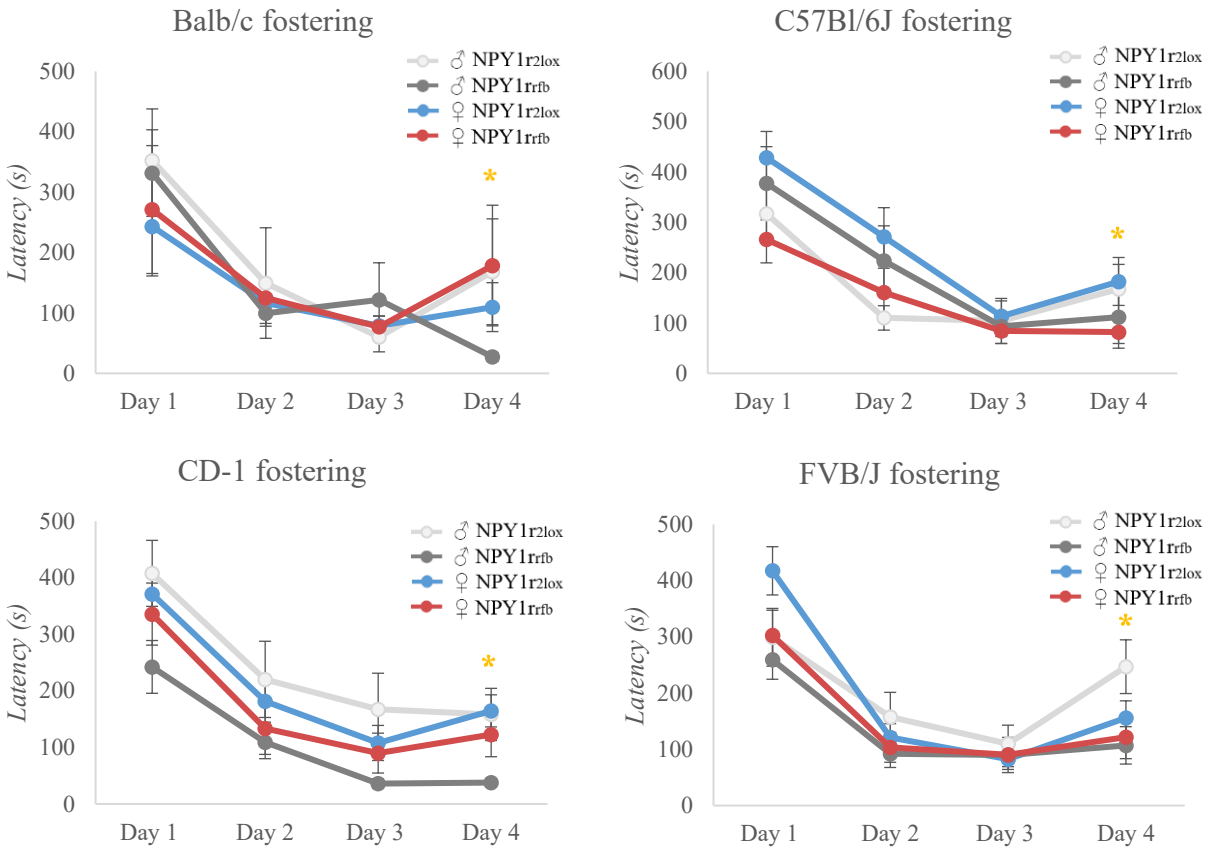


Fig. 4.8 Novelty induced suppression of feeding test. Day 1-3 represent the habituation; Day 4 represent the test in a novel environment. Latency decreased during the habituation. At Day 4 NPY1r^{2lox} males lingered more than NPY1r^{rfb} littermates. Values are presented as mean ± SEM.

4.3.3 Resident-Intruder test

Aggressive behavior was analysed through the Resident-Intruder paradigm. Overall males, regardless the fostering and the genotype, showed more agonistic and aggressive behaviors than females (effect of gender: $F_{(1,155)}$ =13.25480; p<0.001). In particular NPY1r^{2lox} male mice reared by Balb/c mothers showed the highest percentage of time spent in aggressive behavior, when compared with the other groups, while NPY1r^{rfb} male mice reared by C57Bl/6J showed the highest percentage of agonistic behavior. Males reared by CD-1 and FVB/J mothers presented a significant difference between the two genotypes: NPY1r^{rfb} males spent less time in aggressive behavior than their controls. Overall there is an interesting interaction among sex, genotype and adoption ($F_{(3,155)}$=3.20754; p=0.024805).

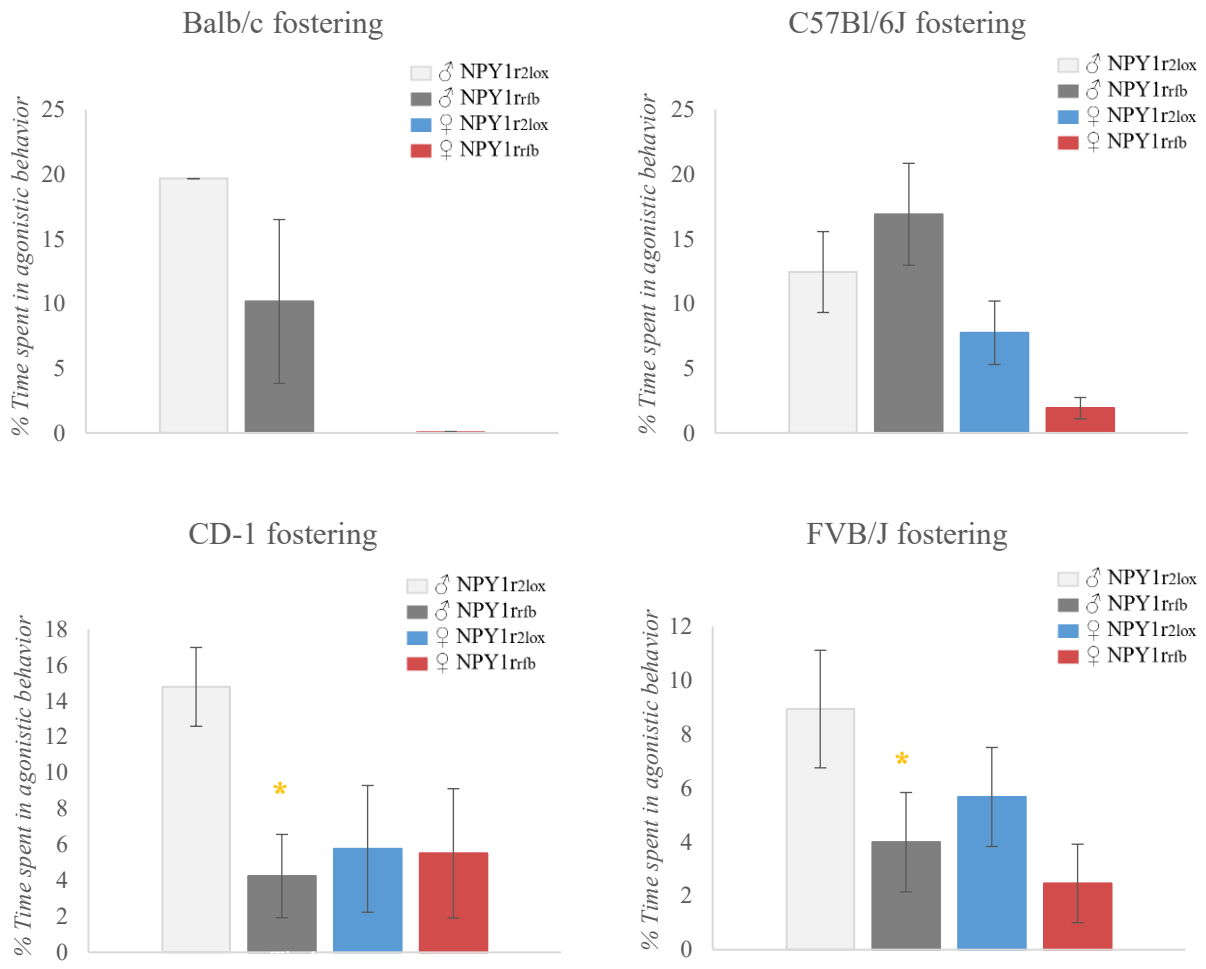


Fig. 4.9 Resident intruder test. Time spent in agonistic behavior. We observed a decrease of the agonistic behavior in NPY1r^{rfb} males when reared by high maternal care dams (FVB/J and CD-1). Values are presented as mean ± SEM.

As expected, latency of attack reflected the percentage of time spent in agonistic behavior: females presented an higher latency, when compared with males (main effect of sex: $F_{(1,240)}$ =11.9453; p<0.001). There was also a strong effect of adoption ($F_{(3,240)}$=10.1135; p<0.001) with animals reared by C57Bl/6J and CD-1 dams that showed a lower latency of attack than the animals reared by other foster dams. We observed an effect of genotype ($F_{(1,240)}$=7.3805; p<0.007073): NPY1r^{rfb} lingered more before attack than their controls. Overall, NPY1r^{2lox} male mice reared by FVB/J and CD-1 dams showed a lower latency of attack when compared with males reared by other groups (interaction among sex, fostering and genotype: $F_{(3,240)}$=3.6411; p=0.013438).

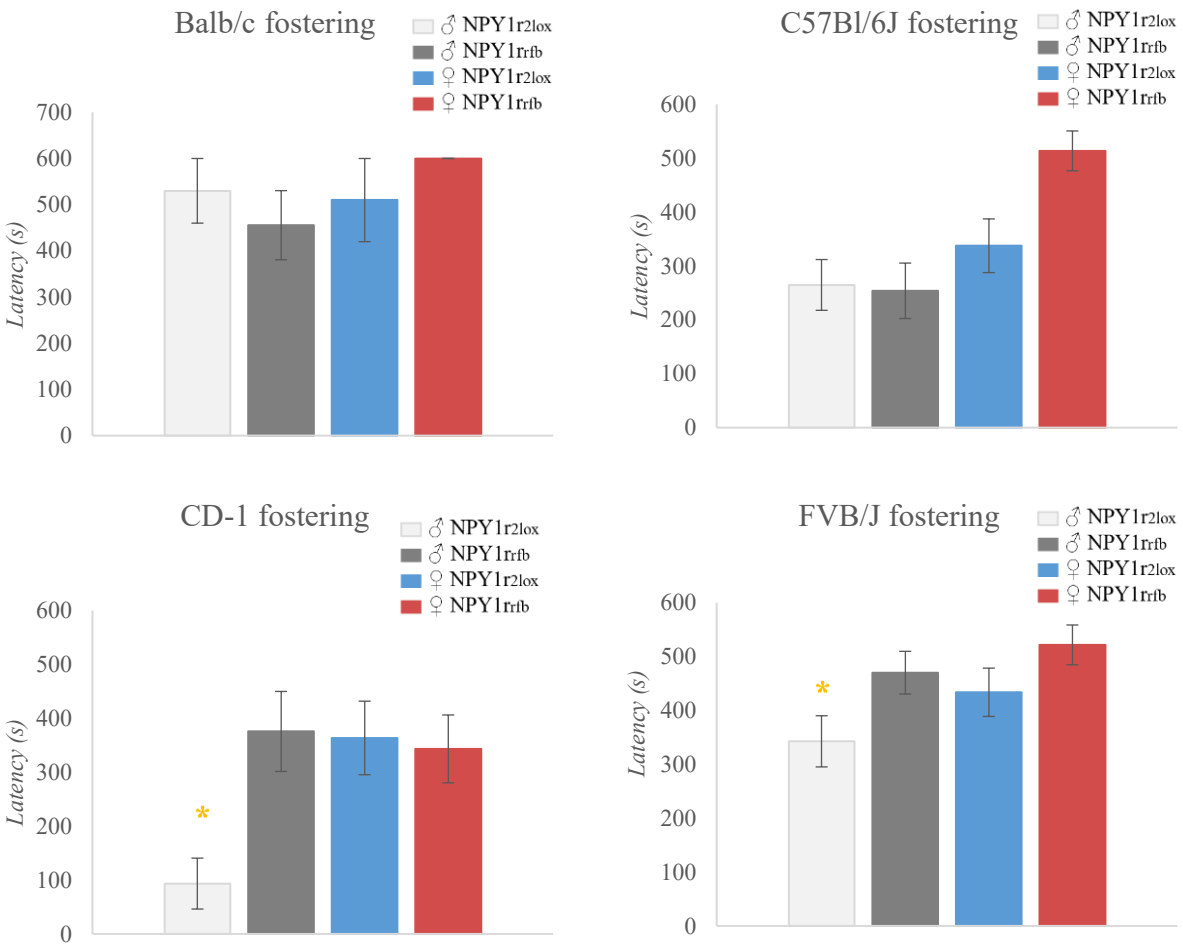


Fig. 4.10 Resident intruder test; Latency of attack. We observed an increase of latency in NPY1r^{rfb} males when reared by High maternal care dams (CD-1 and FVB/J). Values are presented as mean ± SEM.

We decided to investigate social behavior of all the animals with the resident intruder paradigm, analysing the amount of social interaction between subjects. Overall a significant interaction among sex and adoption was highlighted by the statistical analysis ($F_{(3,155)}$=5.0368; p=0.002336): regardless genotype, females reared by CD-1 and Balb/c mothers displayed an higher amount of social behavior than other groups.

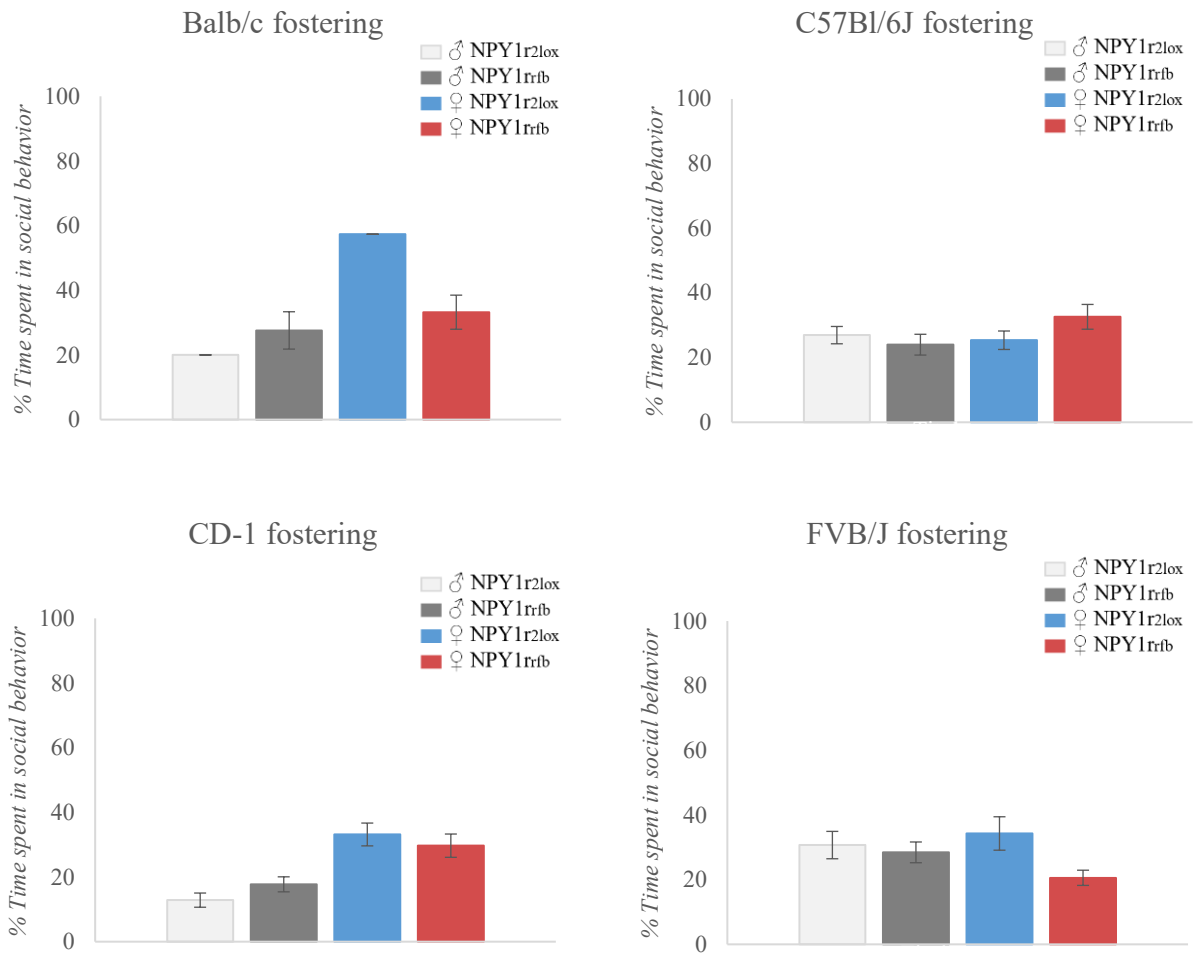


Fig. 4.11 Resident intruder test. Percentage of time spent in social interaction. We observed higher number of social behaviors in females when reared by CD-1 and Balb/c mother compared to the other groups. Values are presented as mean ± SEM.

4.3.4 Body weight and Food Intake

As adult, NPY1r^{2lox} and NPY1r^{rfb} mice of both genders were exposed to standard (STD) or high fat (HFD) diet. Overall male mice, regardless of diet and genotype, showed an higher body weight than females (effect of sex: $F_{(1,205)}$=431.99; p<0.001), even if there is also a strong effect of the diet, with an increase in males body weight during high fat diet (interaction between sex and diet: $F_{(1,205)}$=15.47; p<0.001).The strain of the foster mother seemed to affect body weight: in fact NPY1r^{2lox} and NPY1r^{rfb} C57Bl/6J and FVB/J animals weighted less than the others (effect of the adoption: $F_{(1,205)}$=14.06; p<0.001) during STD, whereas in HFD males reared by FVB/J and CD-1 mothers showed an higher body weight than C57Bl/6J and Balb/c offspring. Overall during days we observed an increase of weight due to the diet and the adoption (interaction among days, adoption and diet: $F_{(30,2050)}$=3.43; p<0.001) but only in males fed with

HFD (interaction among days, sex and diet: $F_{(10,2050)}$=26; p<0.001). As challenged with standard diet animals of both genders didn't show any differences due to genotype ($F_{(1,87)}$=0.520; p=0.653338) but males weighted more than females (effect of sex: $F_{(1,87)}$=169.93; p<0.001) and CD-1 and Balb/c mice, regardless of the sex, were heavier than FVB/J and C57Bl/6J (main effect of the adoption: $F_{(3,87)}$=19.22; p<0.001).

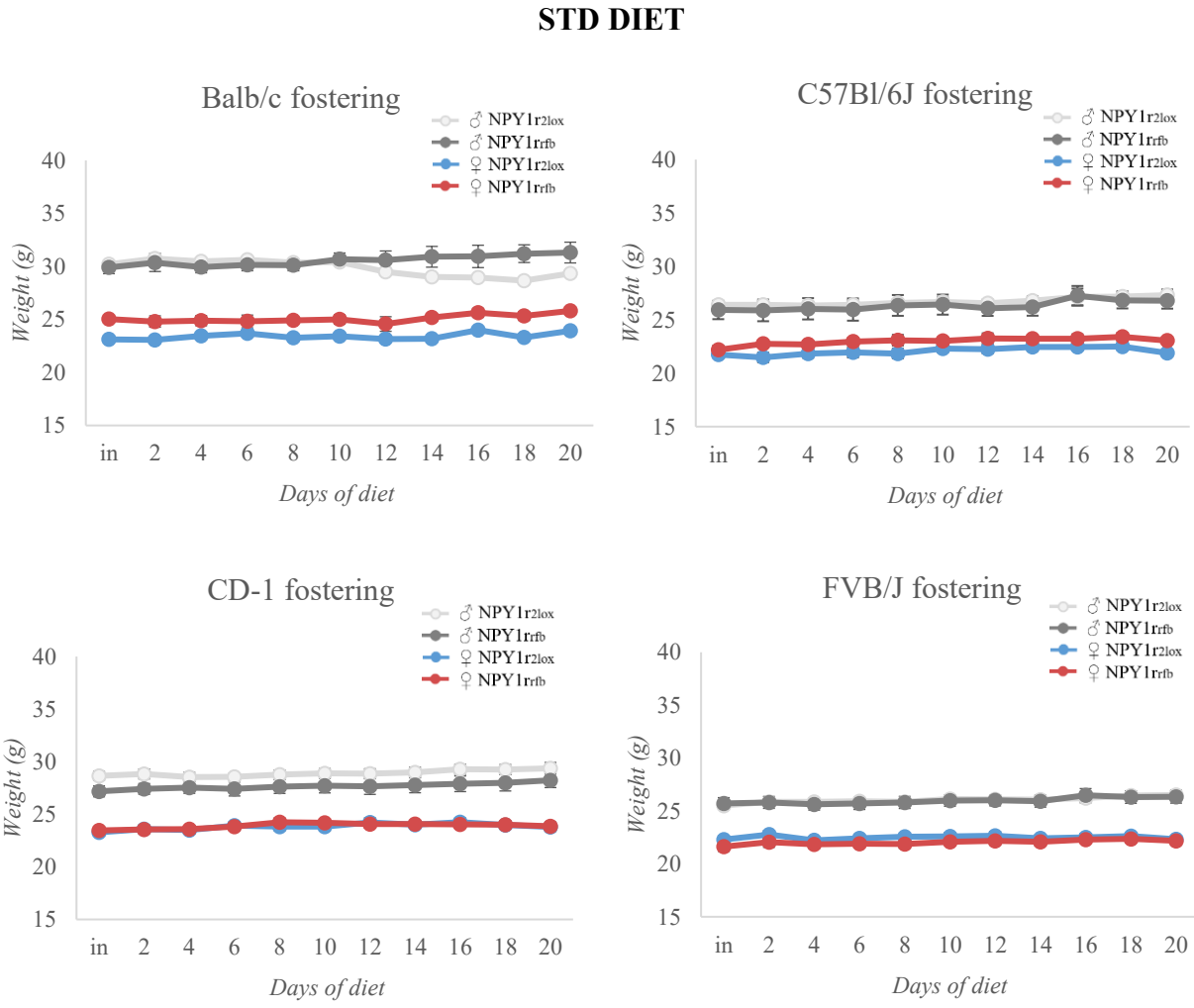


Fig. 4.12 Body weight during the metabolic challenge. Metabolic consequences of STD in NPY1r^{2lox} and NPY1r^{rfb} male and female mice reared by high maternal cares (CD-1 and FVB/J) or low maternal cares (Balb/c and C57Bl/6J) mothers. Regardless of genotype, male mice on STD showed a greater body weight as compared to females. Animals of both genders reared by CD-1 and Balb/c dams showed an increase of body weight when compared to other groups. Values are presented as mean ± SEM.

During HFD males and females showed a strong difference in body weight (main effect of sex: $F_{(1,118)}$=377.63; p<0.001); NPY1r^{rfb} CD-1 males showed an higher body weight when compared to the other groups, and weight increased during days (interaction among days, sex, genotype and adoption: $F_{(30,1180)}$=1.54; p<0.05). Overall, all NPY1r^{rfb} males showed an higher body weight

or a slight increase of weight when compared to their controls, but in males reared by C57Bl/6J NPY1r^{rfb} weighted less than NPY1r^{2lox}.

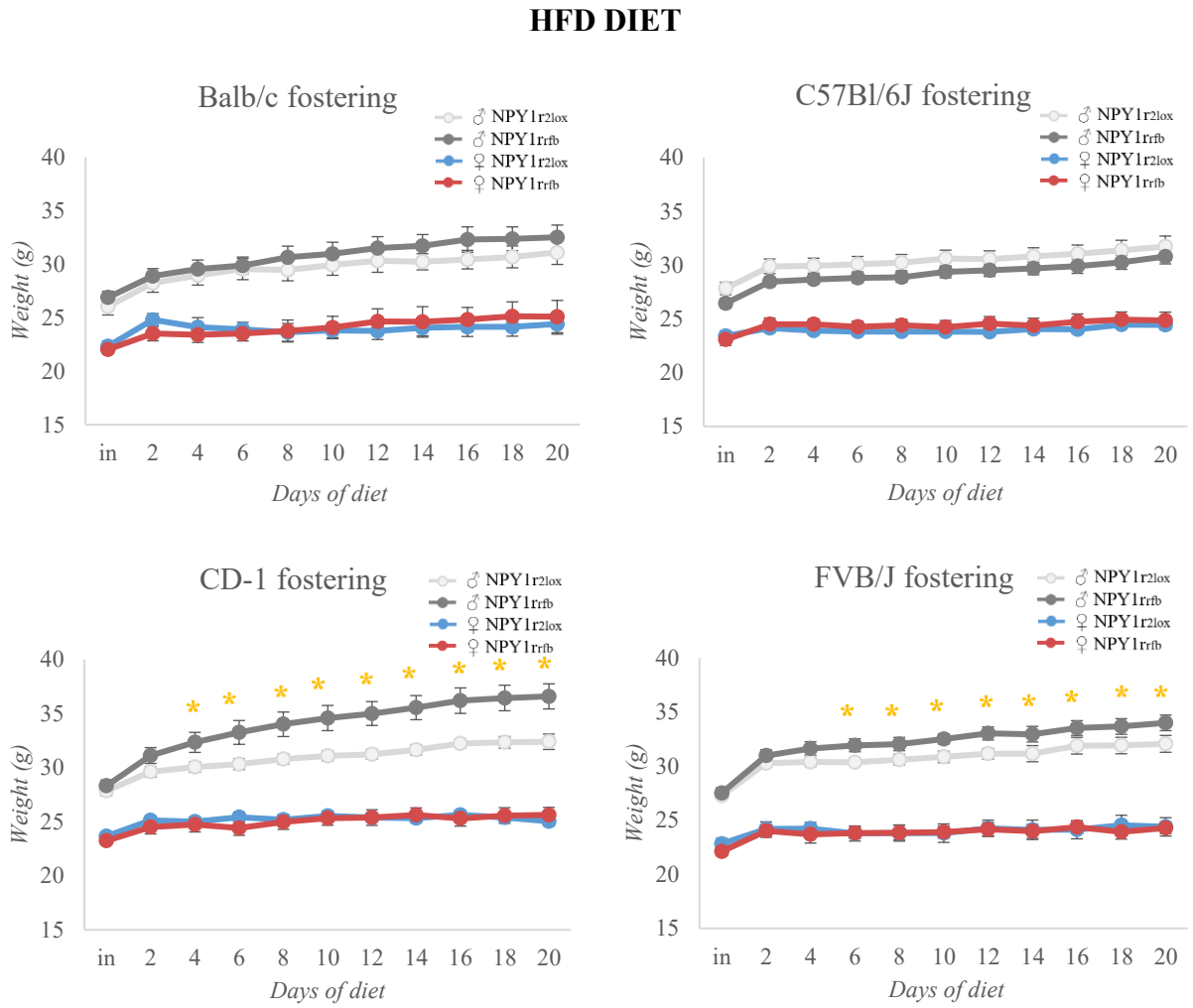


Fig. 4.13 Body weight during the metabolic challenge. Metabolic consequences of HFD in NPY1r^{2lox} and NPY1r^{rfb} male and female mice reared by high maternal cares (CD-1 and FVB/J) or low maternal cares (C57Bl/6J and Balb/c) mothers. Male mice on HFD showed a greater body weight as compared to females. NPY1r^{rfb} males reared by CD-1 dams showed an increase of body weight when compared to their controls. Overall NPY1r^{rfb} males presented a higher weight than NPY1r^{2lox} male mice, except for males reared by C57Bl/6J dams. Values are presented as mean ± SEM.

4.3.5 Glucose tolerance test (GTT)

After the exposure to High fat or Standard diet to evaluate the level of blood glucose at different time points, we performed the Glucose tolerance test (GTT). Overall, sex and diet strongly affected the metabolic response to the glucose challenge with males fed with HFD characterized by increased glycemia when compared with females (main effect of sex and diet: $F_{(1,189)}$=153.621; p<0.001; $F_{(1,189)}$=73.066; p<0.001 respectively; interaction among gender and

diet: $F_{(1,189)}$=16.984; p<0.001). Mice exposed to STD had regular levels of glycemia, confirming and adequate glucose tolerance, with an interaction between time, sex and adoption ($F_{(9,234)}$=1.979; p<0.05). Males fed with STD showed more blood glucose than females, regardless of the genotype (main effect of sex: $F_{(1,78)}$=25.736; p<0.001).

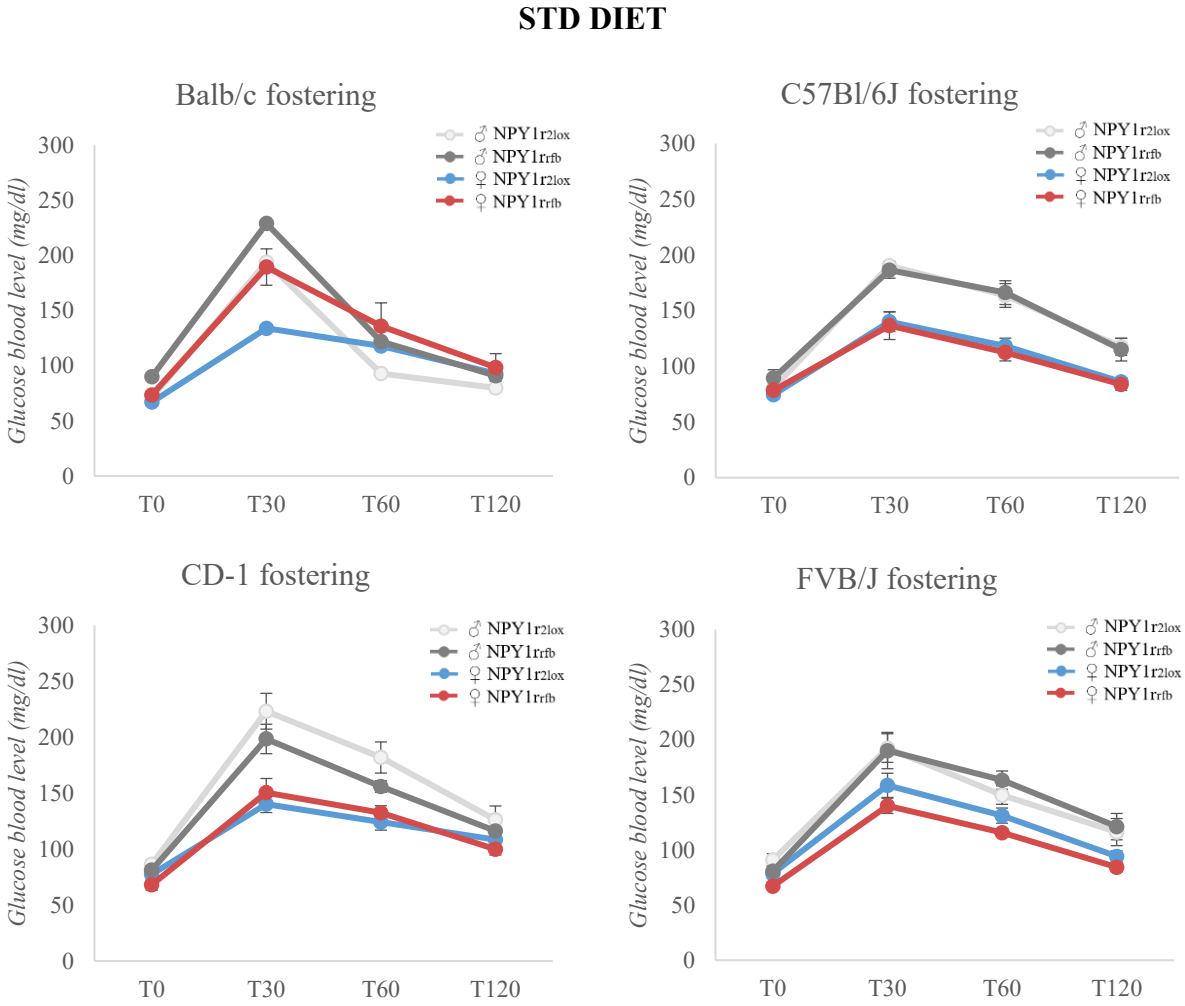


Fig. 4.14 Glucose tolerance test (GTT). Glucose levels during STD in NPY1r^{2lox} and NPY1r^{rfb} male and female mice reared by high maternal cares (CD-1 and FVB/J) or low maternal cares (C57Bl/6J and Balb/c) mothers. Male mice on STD, regardless genotype, showed a higher level of glycemia as compared to females. Values are presented as mean ± SEM.

Exposure to HFD led to higher blood glucose level in both males and females (main effect of sex: $F_{(1,111)}$=214.571; p<0.001), especially NPY1r^{rfb} males had an increase in glycemia (interaction among sex and genotype: $F_{(1,111)}$=6.310; p=0.013446). Above all, NPY1r^{rfb} males reared by CD-1 and FVB/J mothers showed an higher levels of blood glucose when compared to NPY1r^{rfb} with C57Bl/6J and Balb/c mothers (interaction between sex and adoption: $F_{(3,111)}$=5.156; p=0.002248).

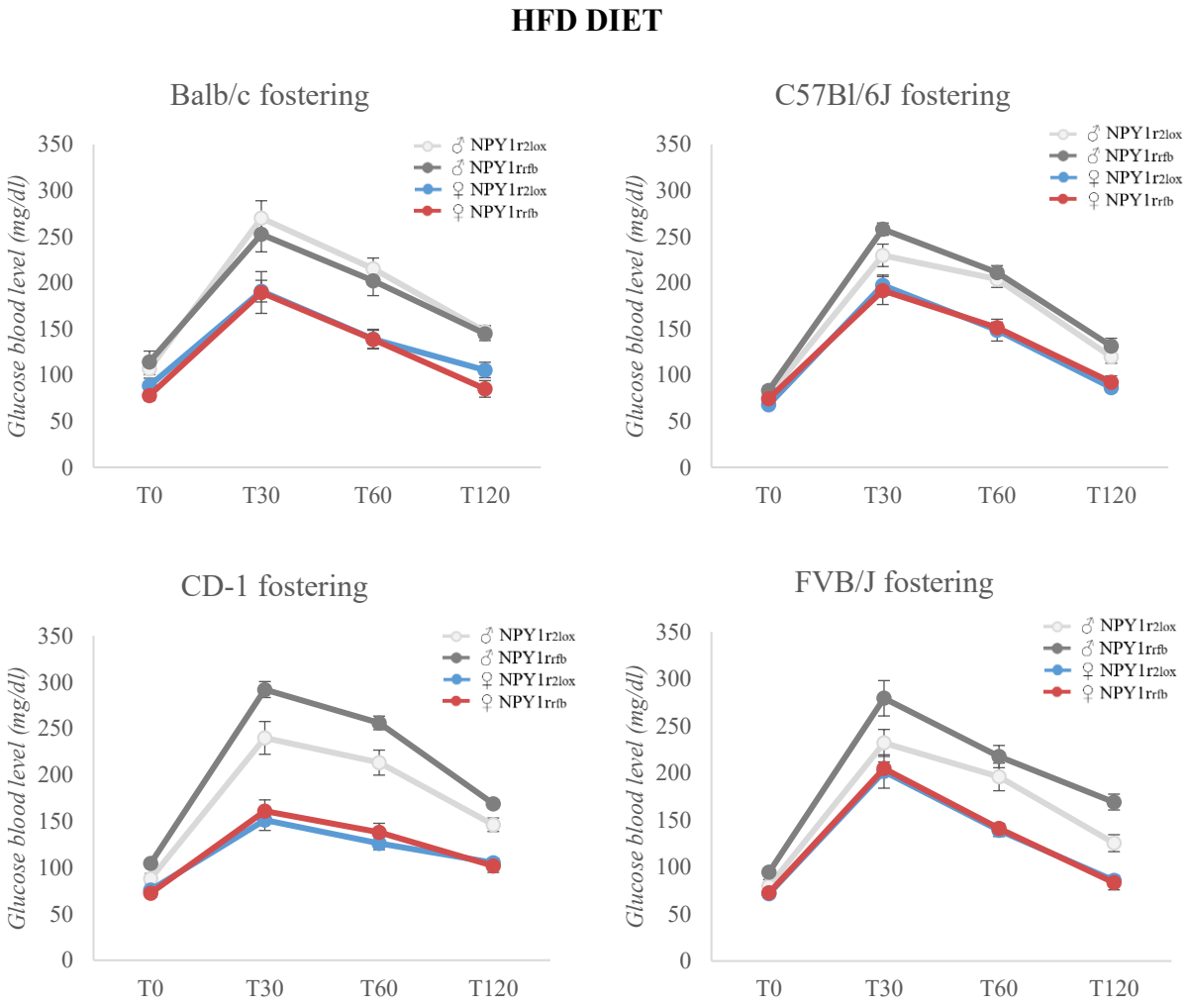


Fig. 4.15 Glucose tolerance test (GTT). Glucose levels during HFD in NPY1r^{2lox} and NPY1r^{rfb} male and female mice. Male mice on HFD, regardless genotype, showed a higher level of glycemia as compared to females. NPY1r^{rfb} male reared by CD-1 and FVB/J mothers presented increased amount of blood glucose, as compared to mice reared by C57Bl/6J and Balb/c mothers. Values are presented as mean ± SEM.

4.3.6 Tissue collection and plasma analysis

As we saw an increase in body weight of mice when fed with HFD, we decided to investigate if there was an increase in visceral adiposity too. At sacrifice we observed a significant abdominal fat weight in HFD fed mice rather than in STD mice (main effect of the diet: $F_{(1,183)}$=75.1388; p<0.001). Overall males showed an higher amount of abdominal white adipose tissue (effect of sex: $F_{(1,183)}$=63.3462; p<0.001) according to body weight measurement during diets, with mice fed HFD presenting an increase in WAT when compared to mice fed with STD (main effect of the diet: $F_{(1,96)}$=87.2007; p<0.001) . Females, regardless of genotype, showed an effect of the adoption: when reared by FVB/J and fed with HFD they had less white adipose tissue than the

other groups (interaction among diet and adoption: $F_{(3,87)}$=2.8669; p<0.05) . NPY1r^{rfb} males fed with HFD and compared with their controls, showed similar amount of WAT when reared by C57Bl/6J or Balb/c mothers (LABN), but when reared by CD-1 and FVB/J (HABN) mothers the levels of abdominal fat increased compared to NPY1r^{2lox} mice (interaction between sex, diet and adoption: $F_{(3,86)}$=4.1370; p=0.008645).

A) **STD DIET**

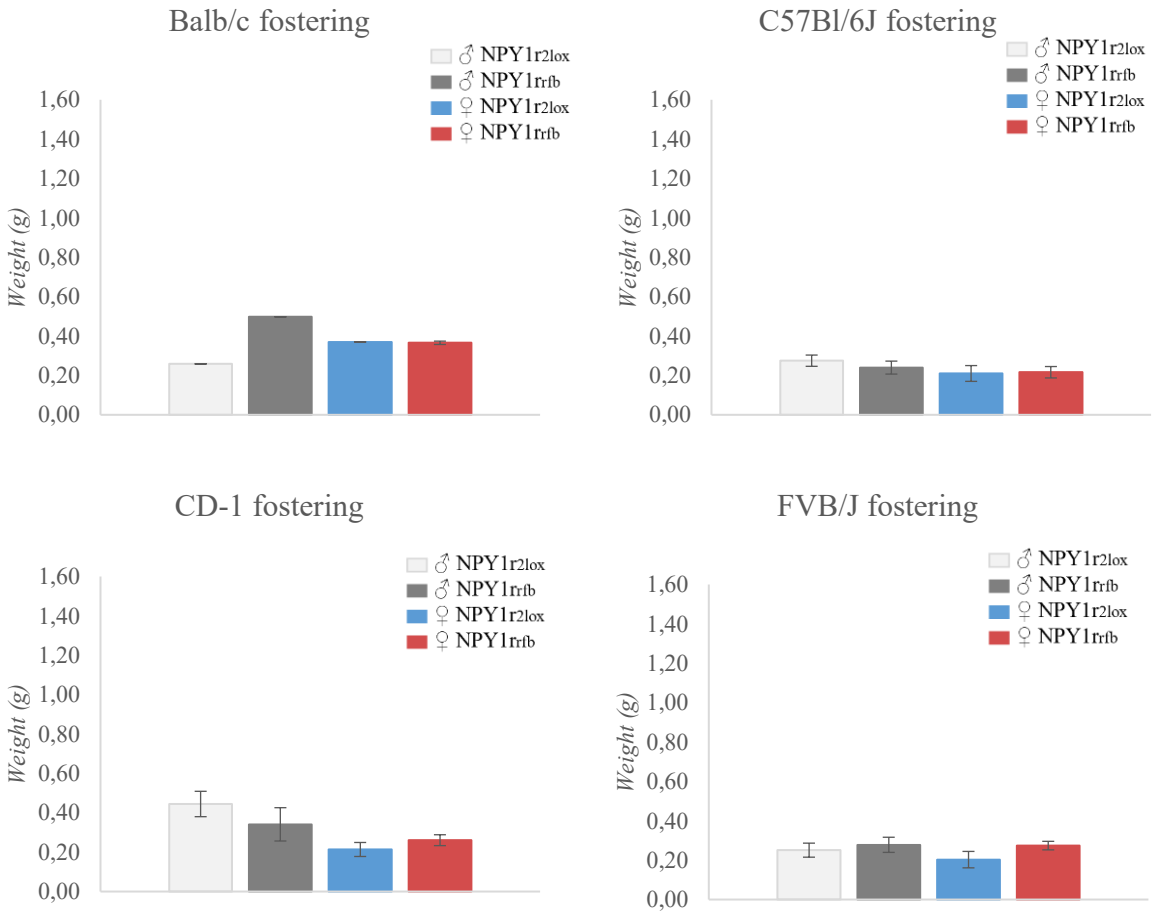


Fig. 4.16 White adipose tissue harvested at sacrifice. A) Amount of WAT in NPY1r^{2lox} and NPY1r^{rfb} male and female mice reared by low maternal care dams (Balb/c and C57Bl/6J) on STD and HFD diet. Male mice on HFD, regardless genotype, showed a higher level of abdominal fat as compared to males in STD. Overall males reared by C57Bl/6J dams and fed with HFD presented an increase in abdominal fat as compared with their respective females. All values are presented as mean ± SEM.

B) **HFD DIET**

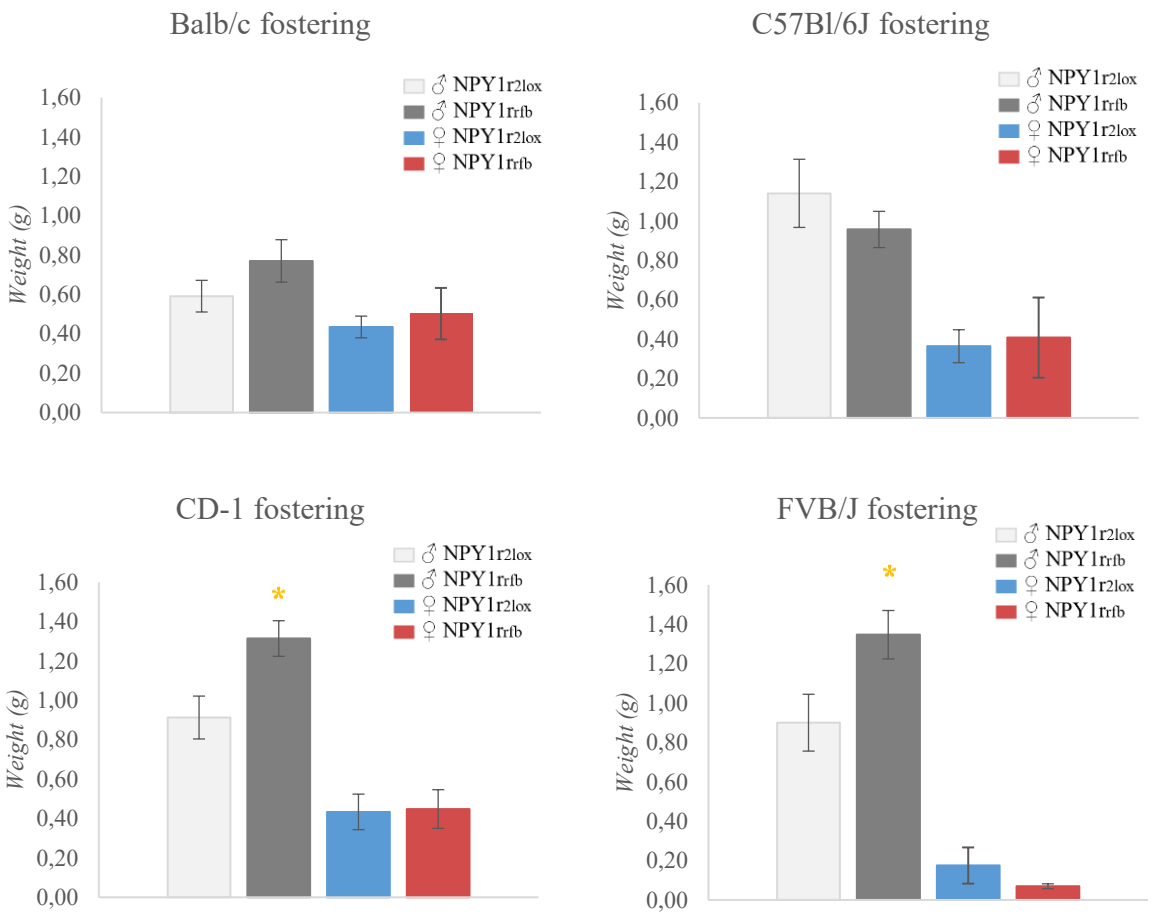


Fig. 4.17 White adipose tissue harvested at sacrifice. B) Amount of WAT in NPY1r^{2lox} and NPY1r^{rfb} male and female mice reared by high maternal care dams (CD-1 and FVB/J) on STD and HFD diet. Overall NPY1r^{rfb} males reared by CD-1 and FVB/J dams presented an increase in WAT when fed with HFD, as compared with females. All values are presented as mean ± SEM.

4.4 Discussion

In this study we assessed the effects of conditional NPY-1r knockout in limbic areas on anxiety-like, aggressive-social behavior and metabolism of male and female mice reared by foster mothers that display different levels of maternal care To this aim we examined adult male and female NPY1r^{2lox} and NPY1r^{rfb} mice reared by high maternal care dams (CD-1 and FVB/J foster dams) and low maternal care dams (Balb/c and C57Bl/6J foster dams).

Overall, our findings show that conditional NPY-1r inactivation induces behavioral effects only in males reared by high maternal care dams by reducing aggressive behavior. Interestingly in females reared by low maternal care dams, especially C57Bl/6J mothers this effect seems to be inverted and NPY1r^{rfb} female mice showed a reduction of the percentage of agonistic behavior. In the Resident/Intruder paradigm NPY1r^{rfb} male mice were characterized by higher attack latency when reared by high maternal care dams and consequently, they spent less time than control mice engaged in agonistic behavior. These results are in apparent contrast to what was described by Karl et al. (2004) who said that germinal ablation of the Y1 receptor gene led to a pronounced increase in territorial aggressive behavior by affecting the serotoninergic system. Moreover, a critical role of Y1-r and 5-HT-1A receptor in integrating behavioral pathways to enabling aggression has been demonstrated (Karl et al., 2004). In NPY-1r conditional knockout mouse model, however, reduced Y1-r expression restricted in the CA1, CA3 and DG areas of the hippocampus induced the opposite response and decreased territorial aggression.

Consistently, NPY1r^{2lox} mice, reared by FVB/J and CD-1 dams, characterized by high expression of NPY-1r in these hippocampal areas showed higher aggressive behavior than NPY1r^{rfb} mice. However, when reared by C57Bl/6J dams the percentage of time spent by NPY1r^{rfb} male mice in agonistic behavior increases, suggesting that levels of maternal care might be a key to better understand aggressive behavior in adults. Anyway, aggressive behavior of NPY1r^{2lox} mice is higher in males reared by CD-1 as compared to males reared by C57Bl/6J. It is apparent that aggressive behavior is influenced by many environmental and experimental variables and further studies are required to clarify the networks involved in this behavior. We already know that female aggression can differ widely based on females' reproductive state and genetic background, and the opponent type.

In our study, as expected, levels of agonistic behaviour in females are significantly lower than males, regardless genotype, but NPY1r^{rfb} females reared by C57Bl/6J dams showed lower percentage of aggressive behavior. Female mice spent most of the time in social investigation: in particular, NPY1r^{2lox} females reared by FVB/J, CD-1 and Balb/c displayed more social behavior than NPY1r^{rfb} females reared by the same foster mothers, while NPY1r^{2lox} females reared by

C57Bl/6J spent less time than the other groups in social investigation. These results are apparent in contrast with literature, in particular Sankoorikal and colleagues, in 2006 proved that C57BL/6J mice showed higher levels of sociability than Balb/c mice when tested for social approach (Sankoorikal et al., 2006). It is possible that these changes in sociability depend on the task used and, in addition to possible environmental factors, the differences in sociability are likely to be because of genetic differences between strains.

Anxiety-like behavior was assessed by means of the Elevated Plus maze (EPM) test and the Novelty Induced Suppression of Feeding (NISF) test. Contrary to what expected, reduced NPY-1r mRNA expression in limbic areas did not affect anxiety-like behavior in the EPM test, but there is an effect of the adoption, as mice reared by Balb/c and FVB/J dams spent less time and showed a lower frequency of entrance in the open space of the apparatus. Overall, both NPY1r^{2lox} and NPY1r^{rfb} males and females reared by high or low maternal care dams spent most of the time in the close arms of the EPM and rarely explored the open arm, suggesting that, regardless of sex, genotype and adoption, they are characterized by an anxious-like phenotype and high trait-anxiety.

In the NISF test, anxiety-like behavior is evaluated through the latency to eat a highly palatable snack in a novel environment. This test is based on the principle that rodents show a clear preference for lipid-rich foods, such as peanuts (e.g. Gaillard et al., 2008) and that the latency to close in and consume a familiar snack in a novel environment is markedly increased in a novel cage with clean bedding (Merali et al., 2003). In this test regardless of the adoption, both male and female NPY1r^{2lox} and NPY1r^{rfb} mice showed a regular daily reduction in the latency to consume the peanut, but the latency only increased in NPY1r^{2lox} male mice when the peanut was presented in a clean bedding-filled cage on day 4. Thus indicate that NPY1r^{rfb} males present lower anxiety-like behavior as compared with their controls: these results contrast with our observation in the EPM, where both control and conditional knockout mice displayed a behavioral profile indicative of high anxiety.

Remarkably NPY1r^{rfb} male mice reared by CD-1 and FVB/J dams showed significantly shorter latency to eat the peanut on all trials as compared to their controls, while NPY1r^{rfb} and NPY1r^{2lox} males reared by Balb/c and C57Bl/6J showed a similar trend. No differences due to genotype or adoption were observed between NPY1r^{2lox} and NPY1r^{rfb} female mice. These data indicate that, regardless the adoption, male and female NPY1r^{2lox} and NPY1r^{rfb} mice are not so sensitive to novelty-induced inhibition of feeding, as they did not show the expected environment-induced avoidance in approaching the highly palatable food. Since these effects are more pronounced in males and especially in NPY1r^{rfb} males reared by HABN mothers, this suggest that a reduction

of hippocampal NPY-1r may affect brain paths involved in the processing of reward-related behavioral responses.

Even if these findings seem to be in contrast with the high level of anxiety observed in EPM it is possible to hypothesize that more than a lower level of anxiety, the behavior displayed by our subjects in the NISF test might be an indicator of an enhanced reward-related response and further studies are needed to test this hypothesis. Otherwise another explanation could be the display of an autistic-like response: in fact the high anxiety displayed in the EPM associated by insensitivity to a novel environment shown in the NISF test might be factors related to autism spectrum disorder (Crawley, 2007). The absence of the inhibition of palatable food intake in novel environment might be interpreted as a resistance to change and response to the changes in routine, characteristics of autism (Crawley, 2007).

In this study we also analyzed the effects of limbic NPY-1r on energy balance and vulnerability to high fat diet induced obesity and metabolic syndrome. Our results shows that reduction of limbic NPY-1r promotes (high fat) diet-induced obesity and partial glucose intolerance in male mice only when reared by dams that showed a high level of maternal care. These findings are supported by the higher body weight, abdominal adipose tissue and blood glucose level observed in male mice with conditional NPY-1r knockout fostered at birth to CD-1 and FVB/J dams.

In female mice reduction of limbic NPY-1r or fostering at birth did not affect body weight growth on a standard diet and did not induce susceptibility to diet induced obesity. Male mice characterized by low limbic NPY-1r expression induced by conditional gene inactivation showed reduced body weight growth, starting when gene deletion was achieved. However, when challenged with exposure to a high fat diet, however, $NPY1r^{rfb}$ male mice, especially when reared by HABN foster mother were more susceptible to develop metabolic disorders.

Although, regardless of the adoption, both $NPY1r^{rfb}$ and $NPY1r^{2lox}$ male mice fed HFD showed increased body weight growth than STD fed groups, $NPY1r^{rfb}$ males showed much greater body weight gain than their control ($NPY1r^{2lox}$) when on HFD. This greater increase in body weight started from the second day of HFD and persisted throughout the 21 days of the diet regimen. Thus, contrary to what reported in males, $NPY1r^{rfb}$ female mice did not show lower body weight growth and when challenged with high fat diet, they didn't develop diet-induced obesity and glucose intolerance. As expected, in male mice, HFD induced an increase of abdominal adipose tissue in both genotypes than the STD groups, regardless of the adoption.

Remarkably, $NPY1r^{rfb}$ on HFD showed much greater WAT than $NPY1r^{2lox}$ mice on HFD only when reared by high maternal care dams (CD-1 and FVB/J), indicating that the increase of body weight showed by $NPY1r^{rfb}$ mice fed HFD might be linked to the high level of white adipose

tissue. Eventually, NPY1r^{rfb} mice on HFD also showed impaired glucose clearance in the glucose tolerance test. Even though HFD did not induce fasting hyperglycemia in either genotype, NPY1r^{rfb} mice reared by high maternal care mothers on HFD showed higher blood glucose level as compared to their controls 30 minutes after the glucose injection, indicating impairment in glucose clearance. This effect is not remarkable in NPY1r^{rfb} vs NPY1r^{2lox} fostered at birth to C57Bl/6J or Balb/c dams.

Taken together, these results suggest that NPY1r^{rfb} male mice, only when reared by mothers that display an high level of maternal behavior, are characterized by a phenotype that predisposes them to develop metabolic disease as demonstrated by their high abdominal adipose tissue and glucose clearance impairment both of which are predictive factors for obesity and metabolic syndrome (Björntorp, 1991a; Jensen, 2008; Ahmed H. Kissebah & Krakower, 1994). In line with our previous study (Bertocchi et al., 2011), the effects of conditional limbic NPY-1r deletion on metabolism are shown in male mice reared by high maternal care dams, which spent most of the time crouching over the pup in the active form of nursing known as arched-back nursing (ABN). Such high proportion of time spent in the ABN posture was consistent during the first postnatal week. According with the results of the previous study (Bertocchi et al., 2011), NPY1r^{rfb} males showed lower body weight growth starting from approximately PND 48, which coincides with the maximal level of NPY-1r gene Cre-mediated inactivation, but this effect is remarkable only in males reared by dams that displayed high amount of maternal care.

Indeed, *in situ* hybridization performed at the end of metabolic challenge on both groups (STD- and HFD-fed mice) showed that NPY-1r mRNA expression in NPY1r^{rfb} mice on STD was reduced in CA1, CA3 and DG as compared to NPY1r^{2lox} mice (Paterlini et al., 2021). This profile of expression is similar to the one observed in male mice reared by FVB/J by Bertocchi and colleagues. After the high fat diet regimen, however, we observed an upregulation of NPY-1r mRNA expression in CA1 and CA3, in NPY1r^{rfb} males fed with HFD and interestingly, the differences in the expression of NPY-1r mRNA between NPY1r^{rfb} and NPY1r^{2lox} disappeared. This finding may suggest that low limbic NPY-1r expression might be responsible for the enhanced predisposition to develop diet-induced obesity and glucose intolerance (metabolic syndrome) in response to high fat diet, which in turn may induce increased expression of NPY-1r mRNA in limbic structures due to compensatory mechanisms. HFD *per se* have has reported to strongly influence synaptic plasticity and gene expression (Lennox et al., 2015; Molteni et al., 2002; Stranahan et al., 2008; Underwood & Thompson, 2016).

While NPY1r^{rfb} males reared by CD-1 and FVB/J mothers and exposed to HFD showed a fast and enduring increase in body weight as compared to controls, NPY1r^{rfb} females seemed to be

"shielded" from the effect of a hyper caloric food. In fact, even when challenged with a HFD, both NPY1r^{rfb} and NPY1r^{2lox} female mice, regardless of the adoption, only showed a slight increase in body weight and a small gain of abdominal adipose tissue as compared to the control group on STD diet. Interestingly, these effects are found regardless of genotype and adoption, suggesting that neither lower expression of NPY-1r in limbic areas and the quality of maternal care are factors promoting metabolic disorders in females. HFD regimen, furthermore, did not promote glucose intolerance in females; NPY1r^{rfb} and NPY1r^{2lox} females on either STD or HFD showed similar blood glucose level curves after glucose administration.

These weaker effects of high fat diet on females may be due to several factors and environmental variables. First of all estrogens have been shown to act like "protectors" against metabolic effects of high fat diet (Litwak et al., 2014; Riant et al., 2009). Riant and colleagues showed that the activation of the estrogen pathway prevents the occurrence and the course of insulin resistance and glucose intolerance induced by high-fat feeding in wild-type mice (Riant et al., 2009). For this reason, estrogens might exert a "shielded" action in NPY1r^{rfb} and NPY1r^{2lox} female, despite the strain of foster mother and the amount of maternal behavior displayed in early postnatal span, that resulted in lower HFD-induced metabolic changes than males.

Conditional deletion of NPY-1r in forebrain excitatory neurons did not affect body weight growth in female mice, although a reduction of NPY-1r mRNA expression was observed in CA1, CA3, DG of NPY1r^{rfb} females as compared to controls. Furthermore, NPY-1r mRNA expression was significantly reduced also in the hypothalamic PVN and medial nucleus of the amygdala (MeA): such a reduction in NPY-1r expression in PVN and MeA was not observed in NPY1r^{rfb} males (Paterlini et al., 2021). Therefore the differential pattern of NPY-1r inactivation in females and males might be responsible for the sex differences observed in the role of limbic NPY-1r in the regulation of body weight.

Based on present and previous studies, we confirm that limbic NPY-1r is differently regulated in males and females and its actions are strongly dependent upon sex. We also demonstrated that, especially in males, the different effects seem to be linked to the early environment and the amount of maternal care received during first postnatal days.

Chapter 5

5.1 Experiment 2: Effects of early maternal environment and limbic NPY-1r expression on behavior and reproduction in NPY1r^{2lox} and NPY1r^{rfb} females

It is known that the conditional knockout of NPY-1r in limbic area exerts different effects on phenotype in males and females. As we observed in experiment described in chapter 5, compared to males, female mice seem to be "resilient" in showing the behavioral and metabolic effects of the conditional deletion of NPY-1r and early post-natal environment. Our hypothesis was that the deletion of NPY-1r can exerts an effect on pregnancy and reproductive behavior. To assess if the conditional deletion of limbic NPY-1r can play a role in reproduction in this experiment NPY1r^{2lox} and NPY1r^{rfb} females reared at birth by Balb/c, C57Bl/6J, CD-1 and FVB/J foster dams (at birth) have been subjected to a reproductive challenge. The experimental outline was as follows: control (NPY1r^{2lox}) and knockout (NPY1r^{rfb}) females were exposed to a reproductive challenge. At PND 120 females underwent Open field test as baseline of anxiety-like behavior. At PND 143 female were mating with C57BL/6J wild-type males. After one week, males were removed from female's cages. At PND 159 female were tested for anhedonic-like behavior, and at PND 160 was performed Open field test (during supposed pregnancy). At PND 161 female underwent the nest building assessment. At pups birth, offspring were sexed and weighed and at PND 4 of pups we performed retrieving test. Maternal behavior of NPY1r^{2lox} and NPY1r^{rfb} females were observed from PND 1 to PND 7.

5.2 Materials and methods

5.2.1 Open field test (OF)

To assess anxiety-like behavior we performed OF test. Animals were placed in the test room at least 3 hours before the beginning of the test (habituation period to new environment and different light). Each animal was placed in a corner of the open arena of polyethylene (53cm x53cm) and left free to explore them for 5 minutes. The arena surface was divided in 3 concentric zones: border, medium and center. Each session of test was videotaped with a camera placed above the arena. All the trials of test were analyzed with an ethological software system (The Ethovision, Noldus, NL). For each subject tested we scored: time spent in each zone of the

arena and the total distance traveled by the animals. To evaluate anxiety level of animals we consider time spent in the center zone. We performed OF test two times: before reproductive event (baseline), and during pregnancy. Between trials arena was cleaned 3 times: with water, with alcohol and finally water.

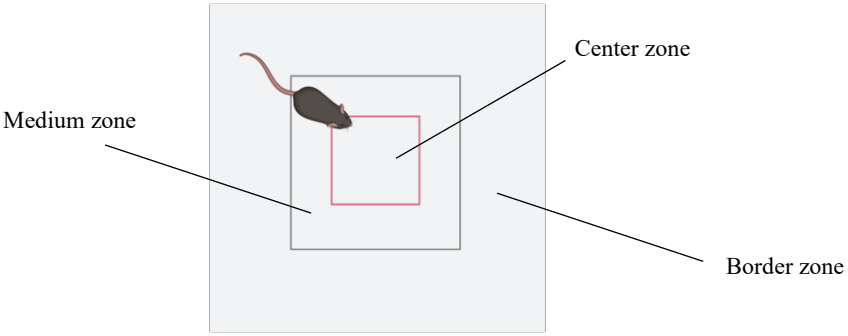


Fig. 5.2 *Open Field Test scheme (Created with BioRender.com)*

5.2.2 Sucrose preference test

Sucrose preference test was used to evaluate anhedonia, the decreased response to rewards, that is associated to depressive state. To evaluate depressive-like behavior during reproductive event, experimental animals were subject to sucrose preference test. Each animal can choose between two bottles placed in their home cage: one of the bottles contain water solution, and the other contain a 1% sucrose solution. All bottles were weighed two times: before the beginning of the test, and 24 hours later. Sucrose preference was expressed as percentage of sucrose solution consumed on the total amount of liquid intake by every experimental animal.

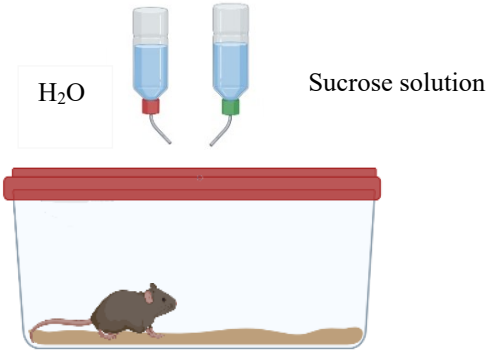


Fig. 5.3 *Sucrose preference test scheme (Created with BioRender.com)*

5.2.3 Nest building assessment test

At PND 160, the day before the expected delivery, we were evaluated nest building of females, as a measure of maternal motivation. The test consists in 3 observations, during 6 hours. At 9:00 AM a pressed cotton square (5cm x 5cm, "Nestlet", Datesand Ltd.) was placed in homecage of every female. Nests were assessed at 11:00 AM, 1:00 PM and 3:00 PM, and we used a rating scale from 1 to 5 to score the nest (Deacon, 2006). Values of rating scale are the following:

- **1** Nestlet not noticeably touched (> 90 % intact)
- **2** Nestlet partially torn up (50-90 % intact)
- **3** Nestlet mostly shredded (> 50% torn up)
- **4** Nestlet torn up, material is gathered into a nest, with walls less than mouse body height (curled up on its side) on 50 % of its circumference.
- **5** Nestlet is totally torn up, with walls larger than mouse body height (curled up on side) on 50 % of its circumference.

Fig. 5.5 *Nest building assessment test scheme (Created with BioRender.com)*

5.2.4 Delivery and body weight pups

We recorded the number of the days from mating (the day in wich male was placed into female's cage) to delivery. Offspring body weight growth was monitored at birth (PND 0) and at PND 6 with a digital balance accurate to 0.01 g (Sartorius, Germany). We recorded also the number of pups in each litter, and reproductive success, defined as the percentage of dams with at least one pup alive at PND 6.

5.2.5 Maternal behavior observation

During the first week of pup's life, we observed spontaneous maternal behavior of knockout and control females. Maternal behavior observation procedure was previous described in "Foster mothers maternal behavior observation" paragraph (Chapter 4).

5.2.6 Retrieving test

To assess maternal motivation behavior and the quality of maternal care, we performed retrieving test at PND 4 of offspring. We removed the mother from her homecage, and we placed pups (males and females separately) in the corner of the cage on the opposite side of the nest. Latency to retrieve pups from the corner of the cage to the nest was recorded. The test has a cut-off of 10 minutes. At the end, all pups were replaced into the nest.

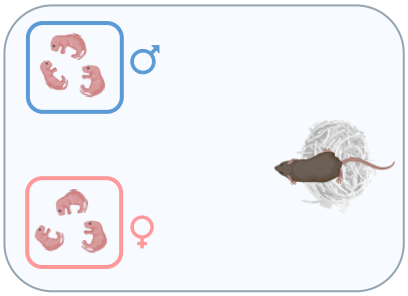


Fig. 5.6 *Retrieving test scheme (Created with BioRender.com)*

5.2.7 Tissue collection

Females were sacrificed by decapitation from PND 6 to PND 12 of pups, following CO_2 exposure. Trunk blood was collected in heparinized tubes (Sarsted) and centrifuge 4000 RPM at 4°C for 10 minutes. Brains were collected and stored at -80°C for later analysis and abdominal adipose tissue (visceral, brown, subcutaneous, perigonadal and retroperitoneal fat) were collected, weighed and frozen in liquid nitrogen for later analysis.

5.2.8 Statistical analysis

A 3-way ANOVA (genotype, adoption and time) for repeated measures was run to analysed the Open Field test. A 2-way ANOVA (genotype and adoption) was run to analysed sucrose preference test, retrieving test, offspring body weight at birth, litter size and spontaneous maternal behavior. When necessary, a post-hoc analysis through Tukey Test was performed. Reproductive success was analysed by means of the Fisher-exact p test. A Wilcoxon matched pairs test was used to evaluate days passed from mating to delivery. Reproductive success was

assessed by means of the Fisher-exact p test. Data were analysed with Statistica 10.0 software (Stat-Soft, Tulsa, OK, USA). Significance was determined when $p<0.05$. Data are presented as mean ± standard error of the mean.

5.3 Results

5.3.1 Open Field Test

We perform Open Field Test in two different times: before and during pregnancy. $NPY1r^{rfb}$ and $NPY1r^{2lox}$ female mice reared by the four different strains of foster mothers were tested for anxiety like behavior. We assessed time spent in each zone of the arena (border, medium and center zone), and total locomotor activity during the test. The cumulative statistical analysis showed that during pregnancy females spent more time in the medium zone of arena compared to the baseline ($F_{(1,102)}$=10,503; p=0,00161), conversely females spent less time in the border zone during pregnancy compared to the baseline ($F_{(1,102)}$=10,479; p=0,00163). No differences were recorded for the time spent in the center zone. Regarding locomotor activity, there's an effect of the strain of foster mothers ($F_{(3,102)}$=10,651; p<0,00001) females reared by CD-1 foster mothers showed the highest levels of locomotor activity and females reared by Balb/c dams showed the lowest levels of locomotor activity of the four strains of adoptions. Moreover, during pregnancy females showed reduced levels of locomotor activity, probably due to the increase in body weight. We also performed separate statistical analysis for each strain of adoption.

$NPY1r^{rfb}$ and $NPY1r^{2lox}$ females reared by BALB/c foster mothers

Statistical analysis carried out on $NPY1r^{rfb}$ and $NPY1r^{2lox}$ female reared by BALB/c foster mothers did not showed differences due to genotype in time spent in border zone, in medium zone and in the center of the arena. On the other hand, there is a difference, regardless the genotype, in total locomotor activity during the test: in detail pregnant $NPY1r^{rfb}$ and $NPY1r^{2lox}$ females showed lower locomotor activity compared to the baseline ($F_{(1,9)}$=9,6407; p= 0,01262).

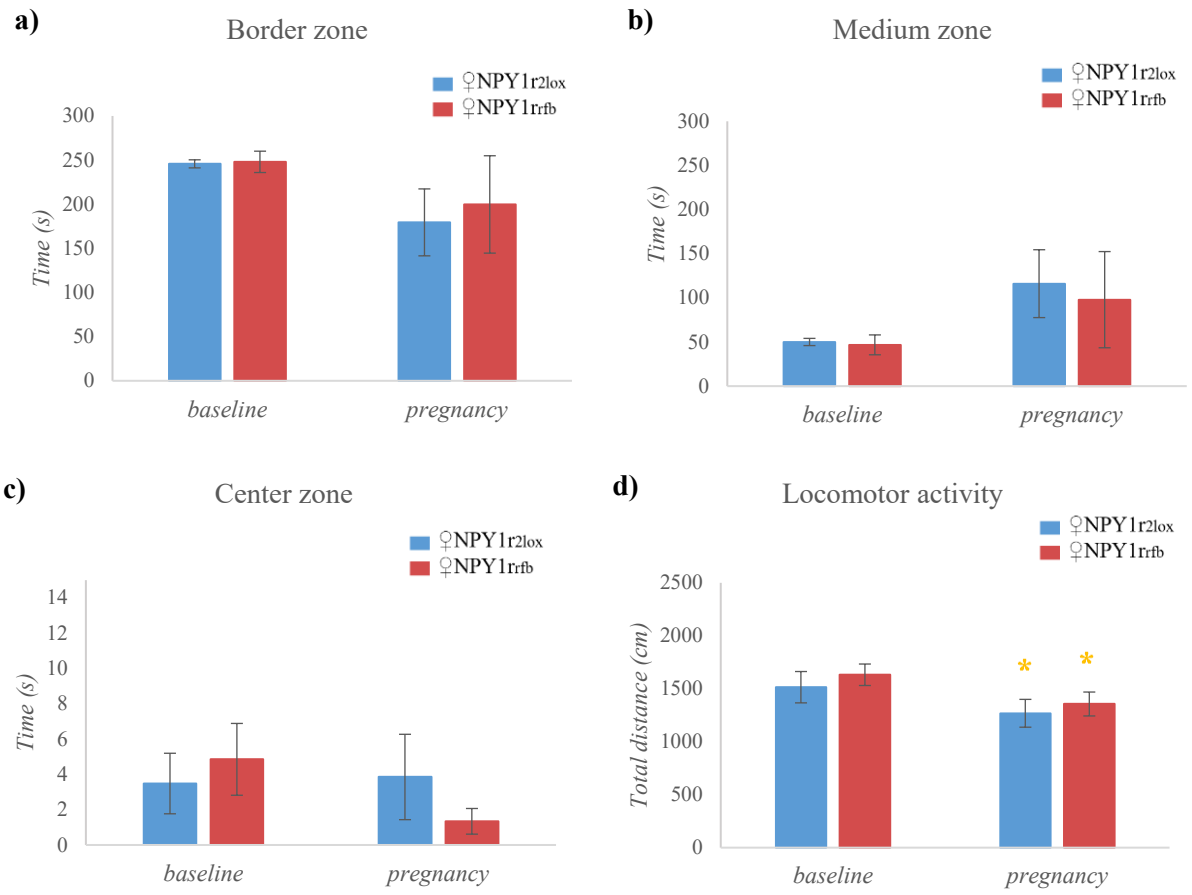


Fig. 5.7 Open field test. a) Time spent in the border, **b)** medium and **c)** center zone of the arena and **d)** total locomotor activity. NPY1r^{rfb} and NPY1r^{2lox} showed decreased in total distance traveled in pregnancy compared to the baseline. Values are presented as mean ± SEM. *indicates a significant effect with $p<0.05$.

NPY1r^{rfb} and NPY1r^{2lox} females reared by C57Bl/6J foster mothers

Females NPY1r^{rfb} and NPY1r^{2lox} reared by C57Bl/6J foster mothers showed less time spent in the border zone and in the center zone during pregnancy (respectively $F_{(1,47)}$=13,117; p=0,00072, $F_{(1,47)}$=6,2315; p=0,01611) compared to the baseline, regardless to the genotype. Conversely, NPY1r^{rfb} and NPY1r^{2lox} females spent more time in the medium zone during pregnancy compared to the baseline ($F_{(1,47)}$=14,696; p=0,00037). Pregnancy involves a reduction in locomotor activity ($F_{(1,47)}$=17,752; p=0,00011) compared to the baseline, regardless to genotype.

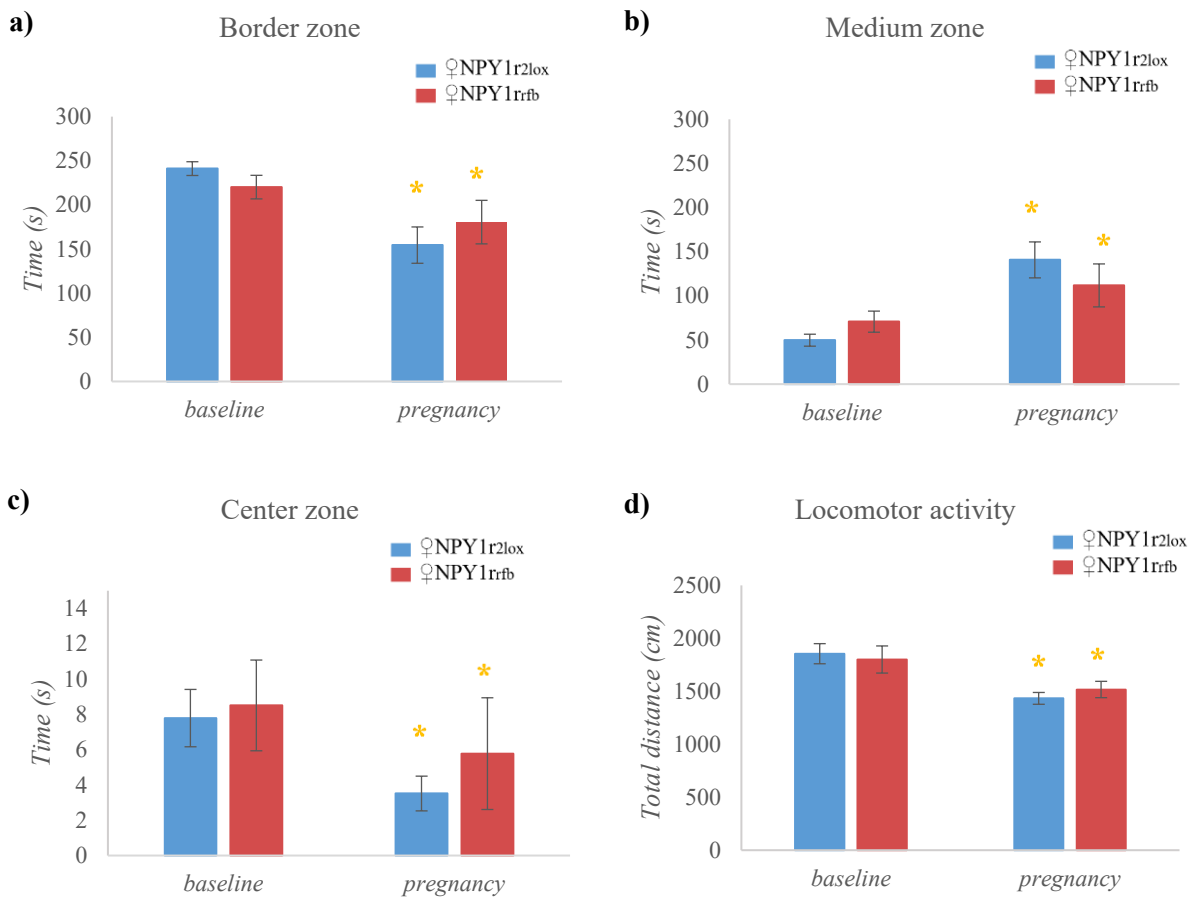


Fig 5.8 Open field test. a) Time spent in the border, **b)** medium and **c)** center zone of the arena and **d)** total locomotor activity. NPY1r^{rfb} and NPY1r^{2lox} showed decreasing time spent in the border and center zone during pregnancy, and an increase in the time spent in the medium zone. Total locomotor activity decreases during pregnancy in both genotypes. Values are presented as mean ± SEM. *indicates a significant effect with $p<0.05$.

NPY1r^{rfb} and NPY1r^{2lox} female reared by CD-1 foster mothers

Statistical analysis carried out on NPY1r^{rfb} and NPY1r^{2lox} females reared by CD-1 foster mothers showed no differences in time spent in the border zone, and medium zone. Interesting, NPY1r^{rfb} females significantly spent less time in the center zone compared to NPY1r^{2lox} females ($F_{(1,16)}$=5,8141; p=0,02828). Pregnant females, regardless to genotype, showed a reduction in locomotor activity compared to the baseline ($F_{(1,16)}$=7,7831; p=0,01311).

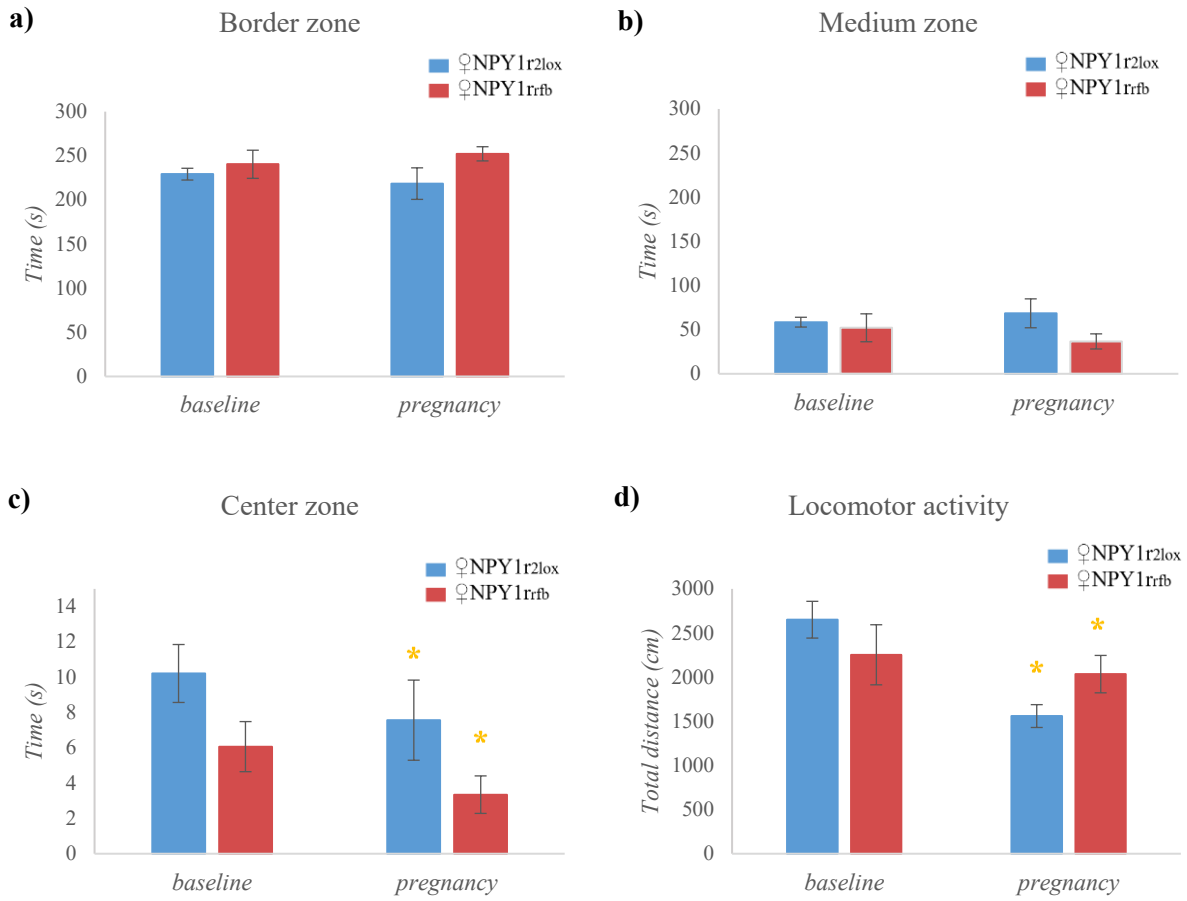


Fig. 5.9 Open field test. a) Time spent in the border, **b)** medium and **c)** center zone of the arena and **d)** total locomotor activity. No difference was recorded in time spent in the border and medium zone. But NPY1r^{rfb} spent less time in the center zone compared to NPY1r^{2lox} females. Total locomotor activity decreases during pregnancy in both genotypes. Values are presented as mean ± SEM. *indicates a significant effect with $p<0.05$.

NPY1r^{rfb} and NPY1r^{2lox} females reared by FVB/J foster mothers

Statistical analysis on NPY1r^{rfb} and NPY1r^{2lox} females reared by FVB/J foster mothers showed no differences in time spent in border zone, medium zone and the center of the arena. However, pregnant females of both genotypes showed a reduction in locomotor activity compared to the baseline ($F_{(1,30)}$= 4,4759; p= 0,04279).

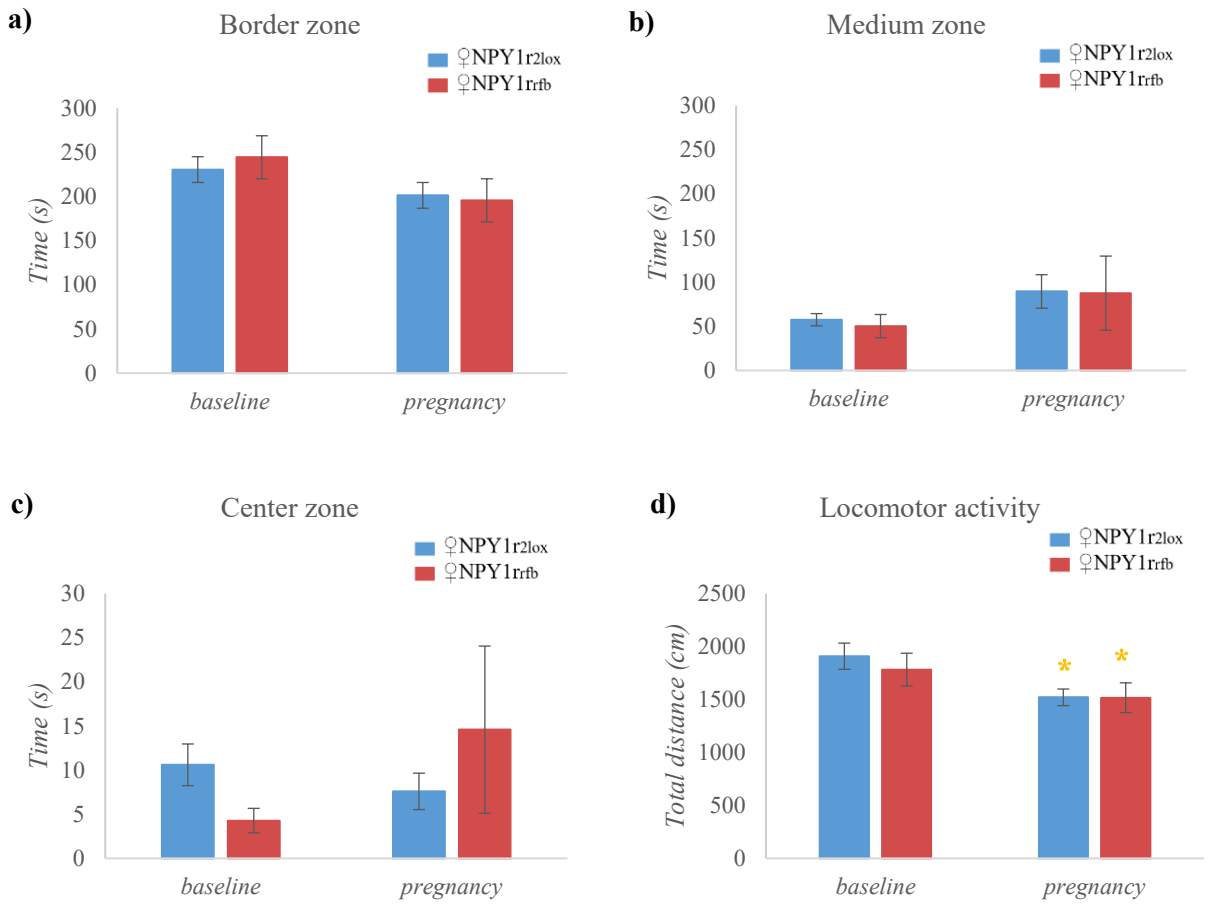


Fig. 5.10 Open field test. a) Time spent in the border, **b)** medium and **c)** center zone of the arena and **d)** total locomotor activity. Regardless to genotype pregnant females spent less time in border zone compared to the baseline. NPY1r^{rfb} females spent more time in the center zone during pregnancy in contrast to NPY1r^{2lox} females that spent less time in center zone during pregnancy. Total locomotor activity decreases during pregnancy in both genotypes. Values are presented as mean ± SEM. *indicates a significant effect with $p<0.05$.

5.3.2 Sucrose preference test

To evaluate depressive-like behavior, we tested NPY1r^{rfb} and NPY1r^{2lox} females from the different fostering. We perform sucrose preference test during pregnancy in order to assess if physiological state of pregnancy can lead to an increase of consumption of sucrose solution rather than water solution. Statistical analysis showed a tendency to significance of the interaction between genotype and adoption ($F_{(3,102}$=2,4385; p=0,068797). More in detail, NPY1r^{rfb} and NPY1r^{2lox} females reared by Balb/c and CD-1 foster mothers showed no differences in consumption of sucrose solution. Conversely, NPY1r^{rfb} females reared by C57Bl/6J and FVB/j foster mothers showed a reduction in sucrose consumption (respectively $F_{(1,46)}$=6,0899; p=0,017376, $F_{(1,29)}$= 5,0408; p= 0,032549) compared to NPY1r^{2lox} females.

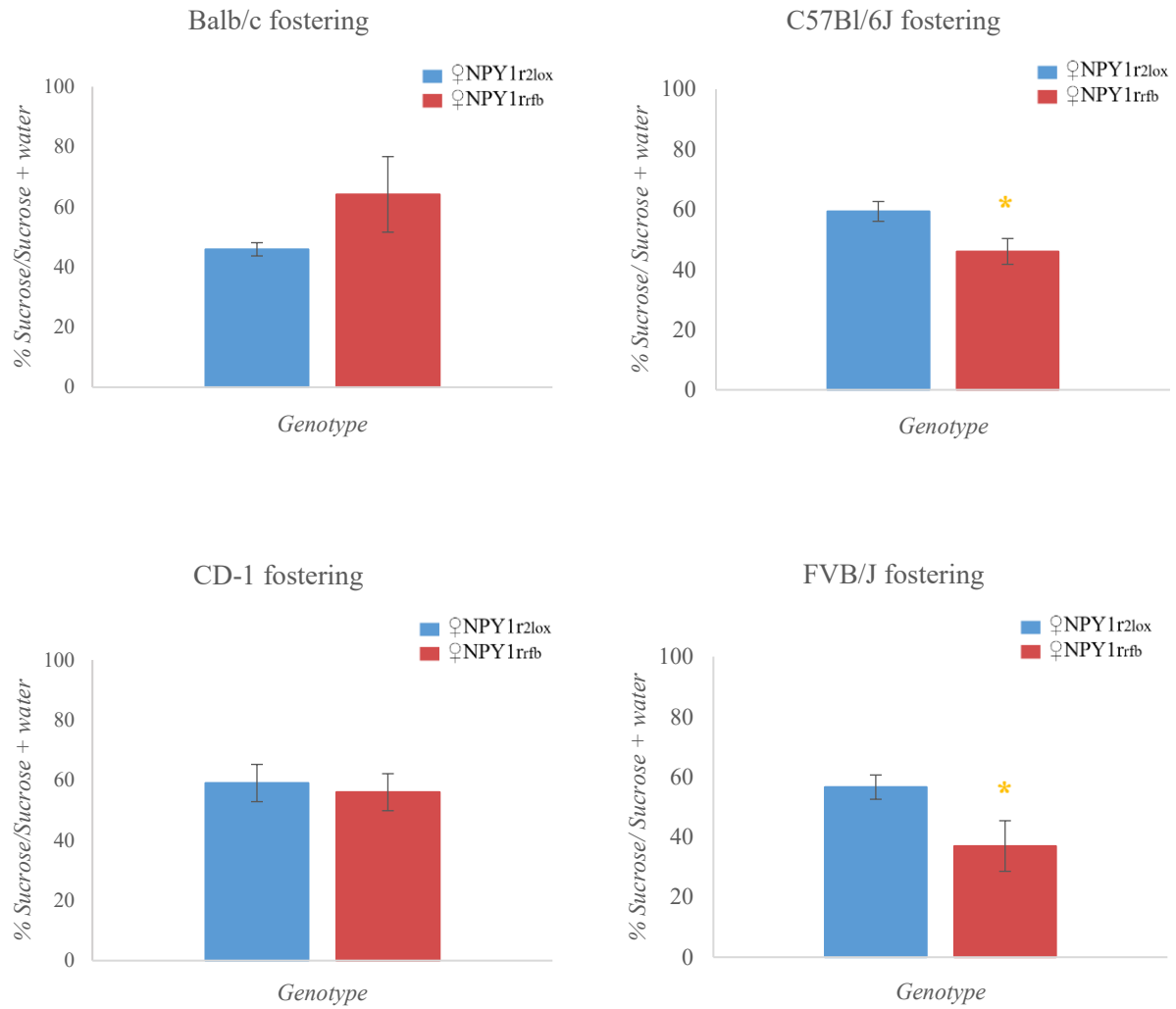


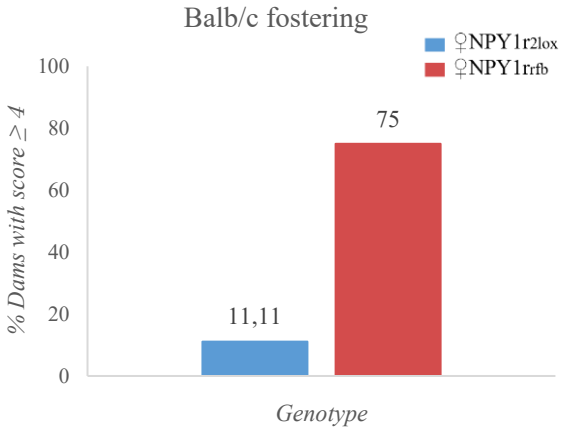
Fig. 5.11 Sucrose preference test. NPY1r^{rfb} females reared by C57Bl/6J and FVB/J strains of foster mothers showed a lower consumption of sucrose solution compared to NPY1r^{2lox} littermates. No differences were recorded in females of both genotypes reared by Balb/c and CD-1 foster mothers. Values are presented as mean ± SEM. *indicates a significant effect with $p<0.05$.

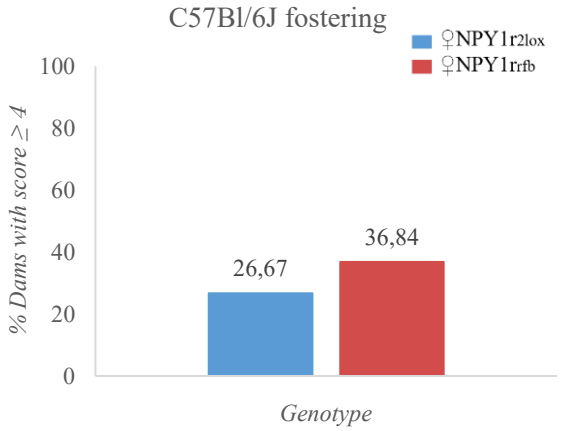
5.3.3 Nest building assessment

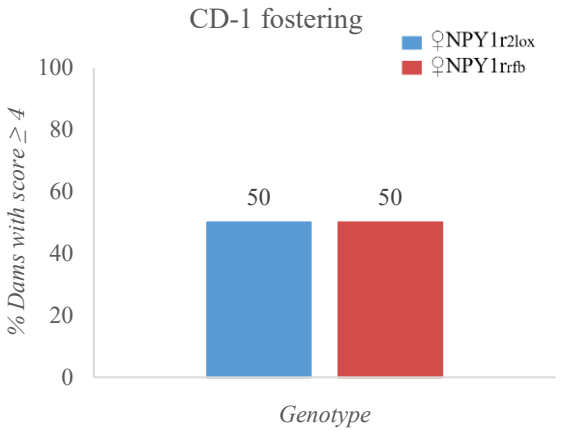
Nest building assessment test was performed the day before the expected delivery. After 2 hours from the nestlet placement, percentage of dams that showed a nest score ≥ 4 was higher in NPY1r^{rfb} females reared by Balb/c dams, compared to the other strain of adoptions although this difference is not statistically significant. The second observation, 4 hours after the nestlet placement, showed higher percentage of NPY1r^{rfb} females reared by Balb/c and C57Bl/6J dams with a nest score ≥ 4, compared to females belongs to CD-1 and FVB/J adoptions. The last observation, 6 hours after nestlet placement, revealed a significative effect of genotype in C57Bl/6J adoption (Fisher exact test value= 0.0387; $p<0.05$), with an higher percentage of NPY1r^{rfb} females reached a nest score ≥ 4. Even though there's no statistical significance,

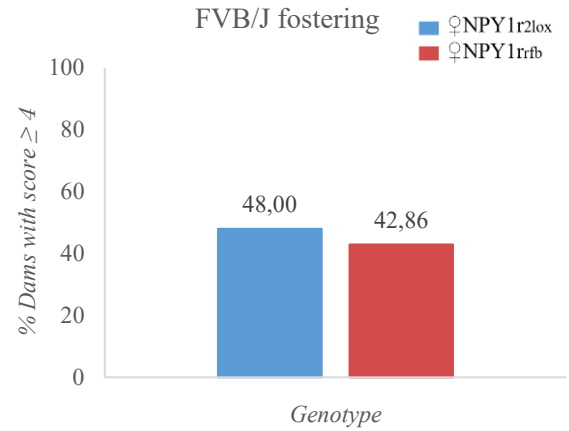
females reared by Balb/c mothers showed the same trend of C57Bl/6J fostered females. No difference were recorded in CD-1 and FVB/J females.

2 hours assessment

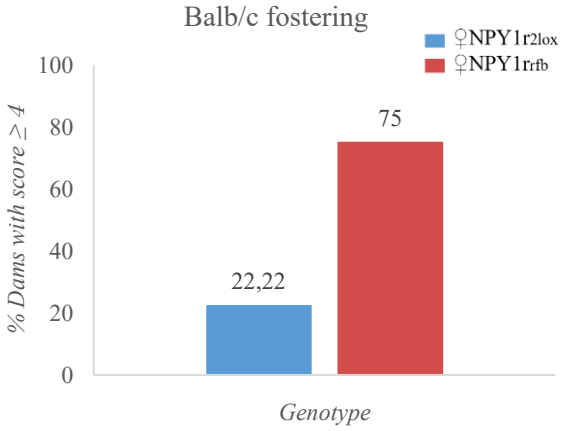


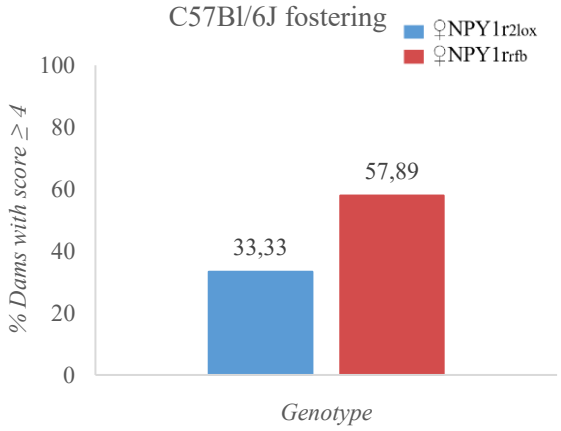


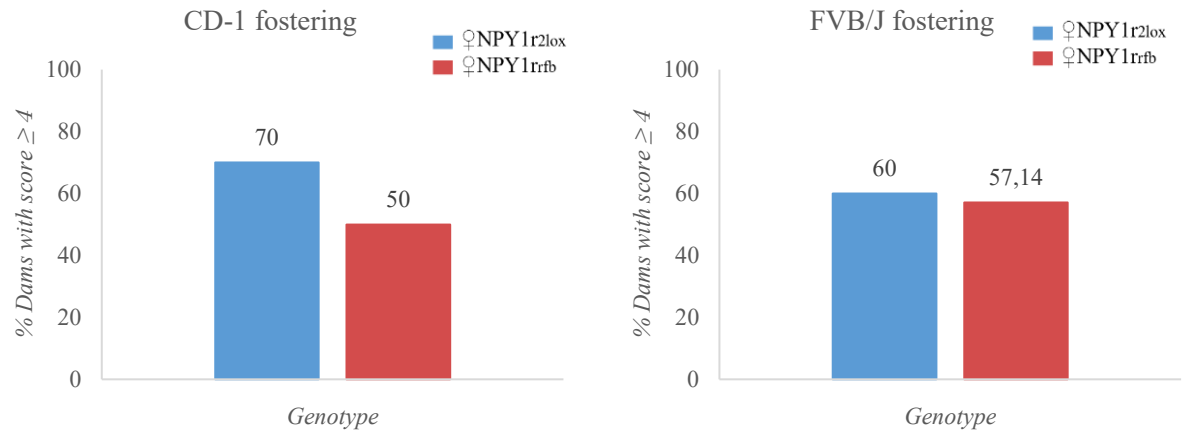




4 hours assessment







6 hours assessment

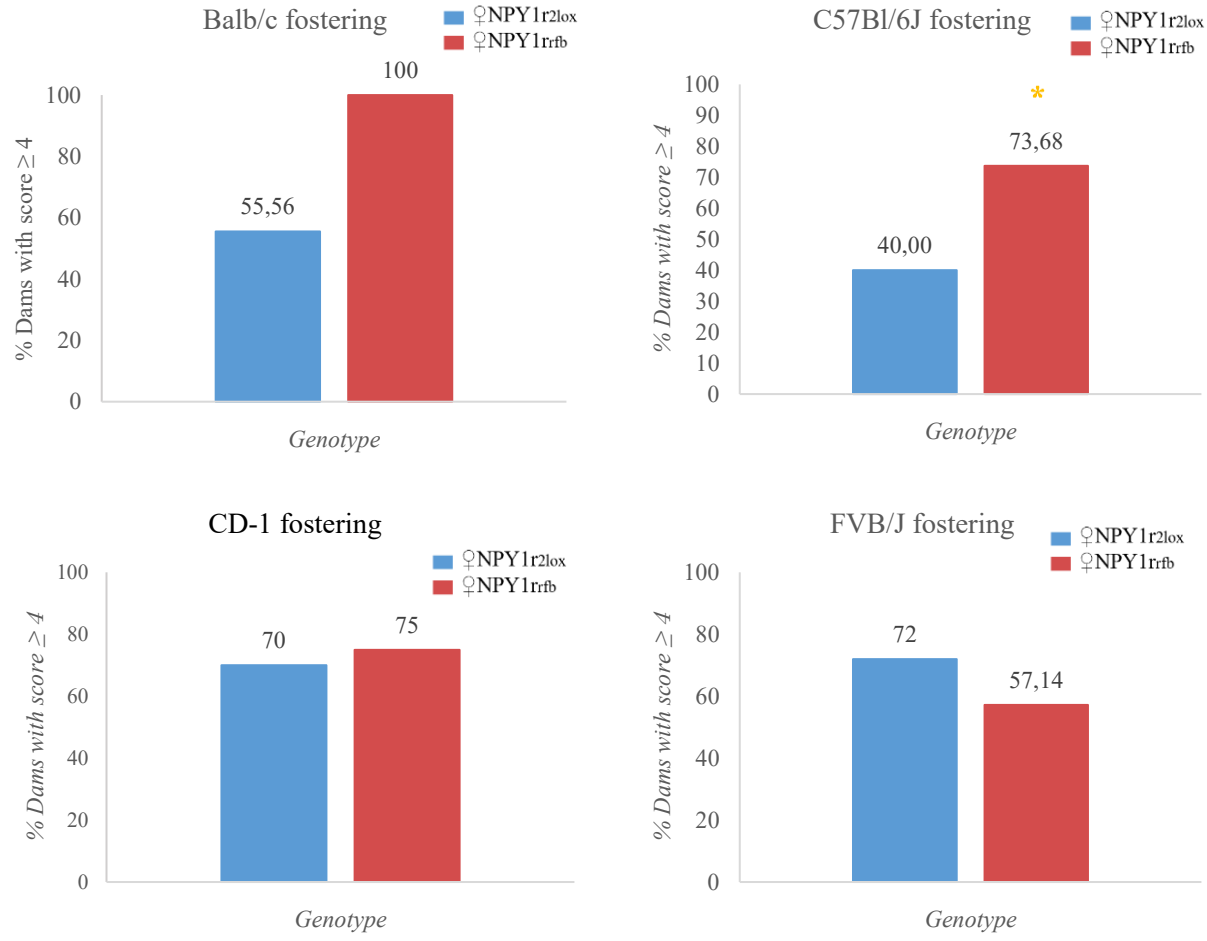


Fig. 5.12 Nest building assessment. 2 hours, 4 hours and 6 hours assessment, % of dams with score ≥ 4. A higher percentage of NPY1r^{rfb} dams reared by Balb/c and C57Bl/6J foster mothers showed a nest score ≥ 4 compared to NPY1r^{2lox}. *indicates a significant effect with $p<0.05$.

5.3.4 Delivery and body weight of pups

Days from mate to delivery

Days from mating to delivery were recorded for NPY1r^{rfb} and NPY1r^{2lox} females. The mating day is defined as the day in which males were placed in the same cage with females. And the day of delivery is the day in which females gave birth to pups. Data not showed differences in the number of days in females of both genotypes reared by Balb/c, C57Bl/6J and FVB/j foster mothers. Indeed, NPY1r^{rfb} females reared by CD-1 foster mothers showed a lower number of days from mate to delivery compared to NPY1r^{2lox} females (effect of genotype: $F_{(1,64)}$= 6,9350; p= 0,01059).

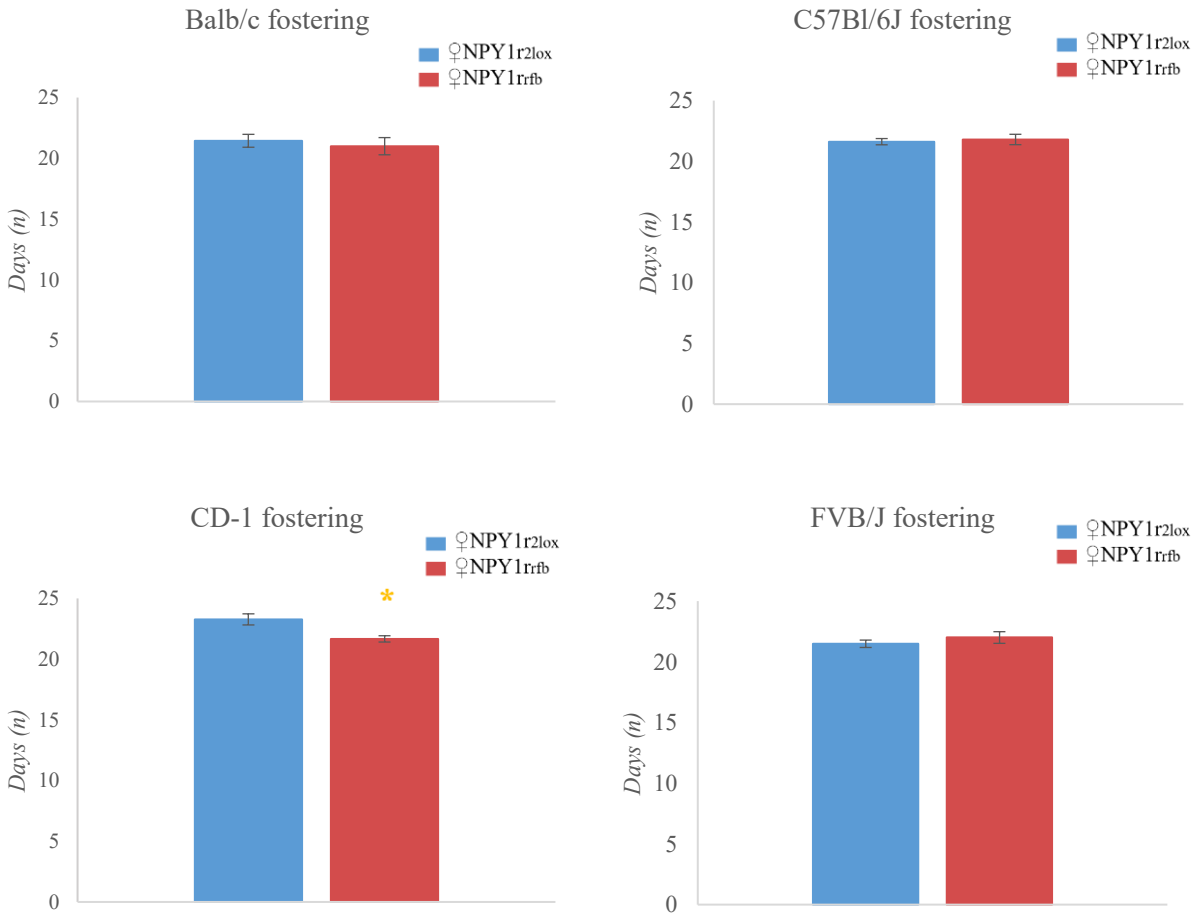


Fig. 5.15 Days from mate to delivery. NPY1r^{rfb} females reared by CD-1 foster mothers showed a lower number of days from mate to delivery compared to NPY1r^{2lox} females. No differences were observed in the other groups. Values are presented as mean ± SEM. *indicates a significant effect with p<0.05.

Number of pups

At birth, offspring of NPY1r^{rfb} and NPY1r^{2lox} females were sexed and weighed. The analysis of the number of pups in each litter showed no differences due to genotype or strain fostering of females.

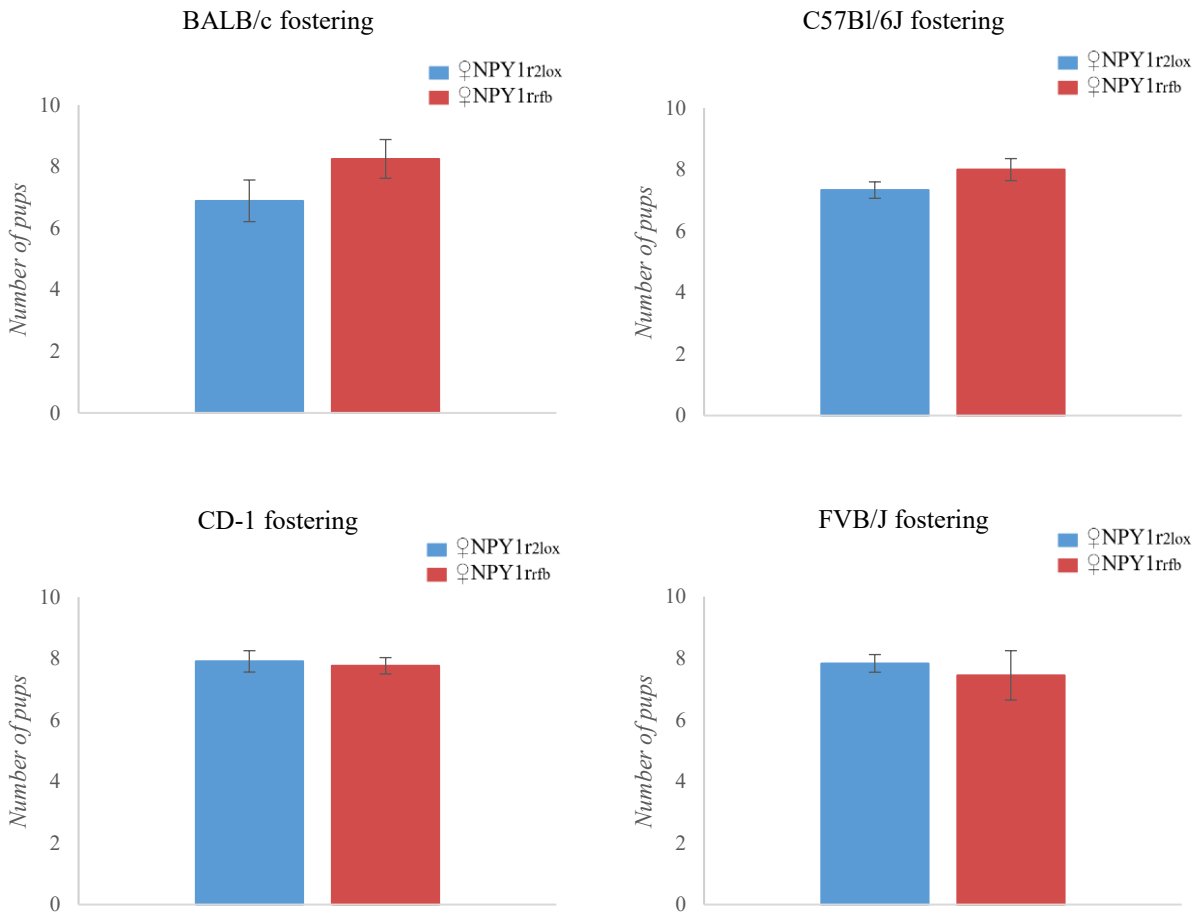


Fig. 5.16 Number of pups. No differences were observed in both genotypes and strain of adoption. Values are presented as mean ± SEM.

Despite no differences was recording in the number of pups delivered, NPY1r^{rfb} pups showed lower body weight compared to NPY1r^{2lox} pups, the trend is the same for females from the four different adoptions. In particular, statistical analysis revealed a significant effect of dam's genotype on body weight of pups ($F_{(1,176)}$=10,880; p=0,00118); NPY1r^{rfb} pups weighed less compared to NPY1r^{2lox} pups. When we analysed apart the four strain of foster mothers we observed no significant effect of genotype in NPY1r^{rfb} and NPY1r^{2lox} reared by Balb/c foster

mothers (probably due to the small number of experimental subjects), whereas NPY1r^{rfb} pups showed lower body weight when reared by C57Bl/6J ($F_{(1,64)}$= 7,7611; p=0,00702), CD-1 ($F_{(1,64)}$= 8,3960; p= 0,00514) and FVB/J ($F_{(1,37)}$= 6,9406; p= 0,01223) foster mothers, compared to NPY1r^{2lox} pups.

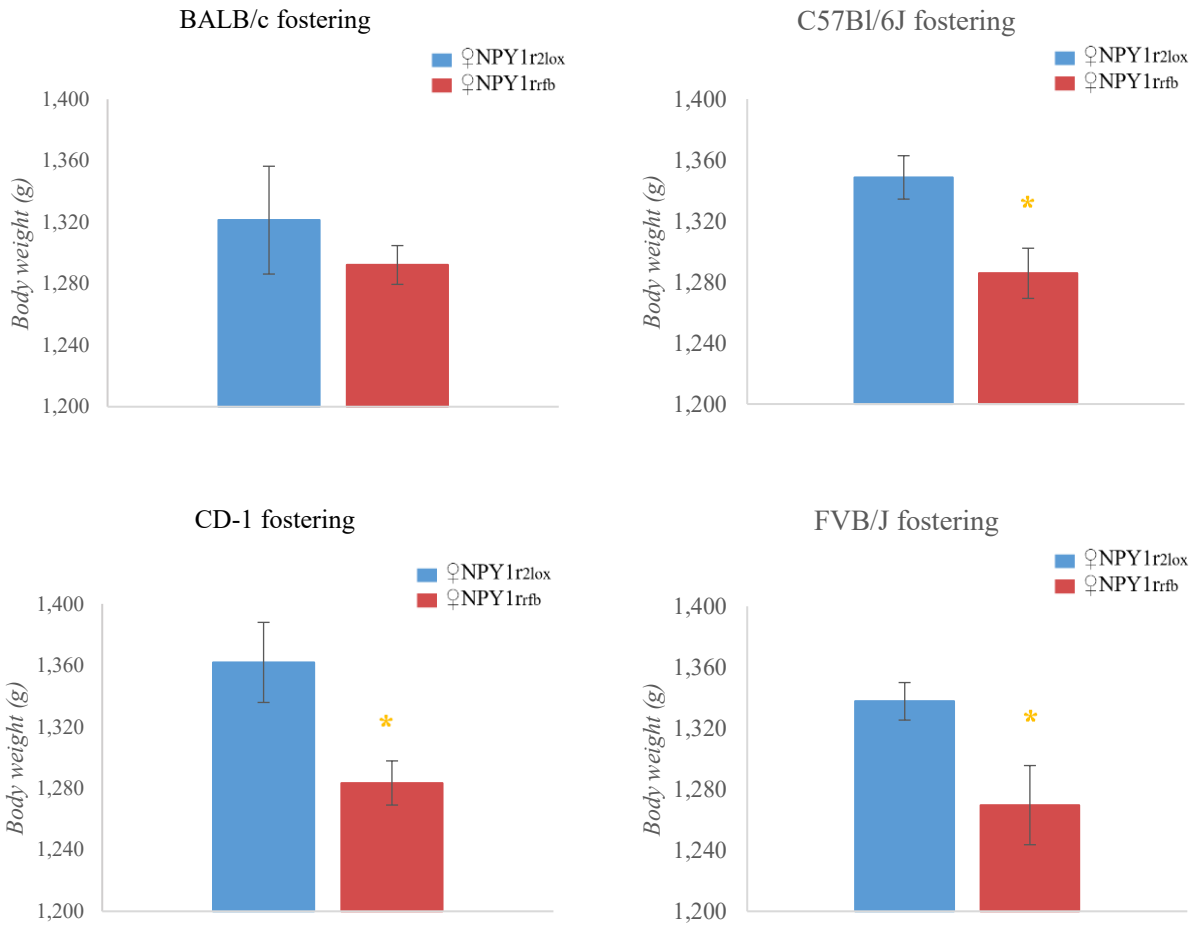


Fig. 5.17 Body weight of pups. Analysis of body weight of pups showed that NPY1r^{rfb} offspring from C57Bl/6J, CD-1 and FVB/J fostering showed a lower body weight compared to NPY1r^{2lox} offspring. Values are presented as mean ± SEM. *indicates a significant effect with p<0.05.

Reproductive success

We define reproductive success as the number of females (expressed as percentage) with at least one pup alive, one week after delivery (PND 6). Statistical analysis suggests a significant effect of genotype, as shown in pictures NPY1r^{rfb} females showed a reduction in reproductive success compared to NPY^{2lox} females regardless to the strain of foster mothers. More in detail, the values of Fisher Exact Test were 0,0517; p< 0,1 for NPY1r^{rfb} reared by Balb/c, 0,0002; p<0,01 for

NPY1r^{rfb} reared by C57Bl/6J, 0,0009; p<0,01 for NPY1r^{rfb} reared by CD-1 and 0,0009; p< 0,01 01 for NPY1r^{rfb} reared by FVB/J foster dams.

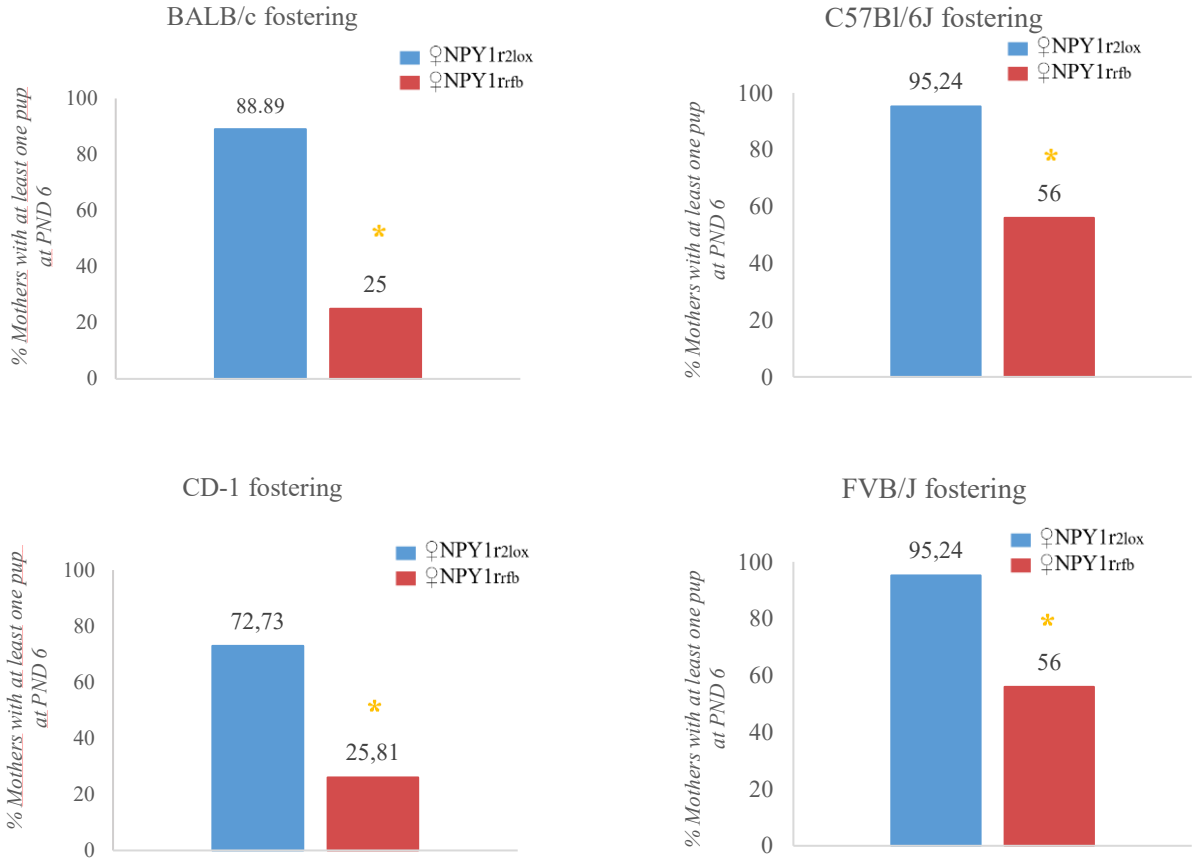


Fig. 5.18 Reproductive success. NPY1r^{rfb} showed lower reproductive success compared to NPY1r^{2lox} offspring regardless to the strain of adoption. We define reproductive success as the % of the dams with at least one pup alive at PND 6. *indicates a significant effect with p<0.05.

5.3.5 Maternal behavior

A 3-way ANOVA (genotype, adoption and days) for repeated measures was conducted on maternal behavior observation of NPY1r^{2lox} and NPY1r^{rfb} dams reared by Balb/c, C57Bl/6J, CD-1 and FVB/J foster mothers. Statistical analysis showed an effect of days on several behavior, with an increase during the days of out of nest activity, eating, and active behavior, and a decrease during days in pup-related behavior (arched back nursing, nursing and licking pups). Furthermore there's an effect of adoption on nursing ($F_{(3, 60)}$= 2,9933; p=0,03780) with females reared by CD-1 dams that showed higher weekly percentage of nursing behavior compared to the others strain of adoption.

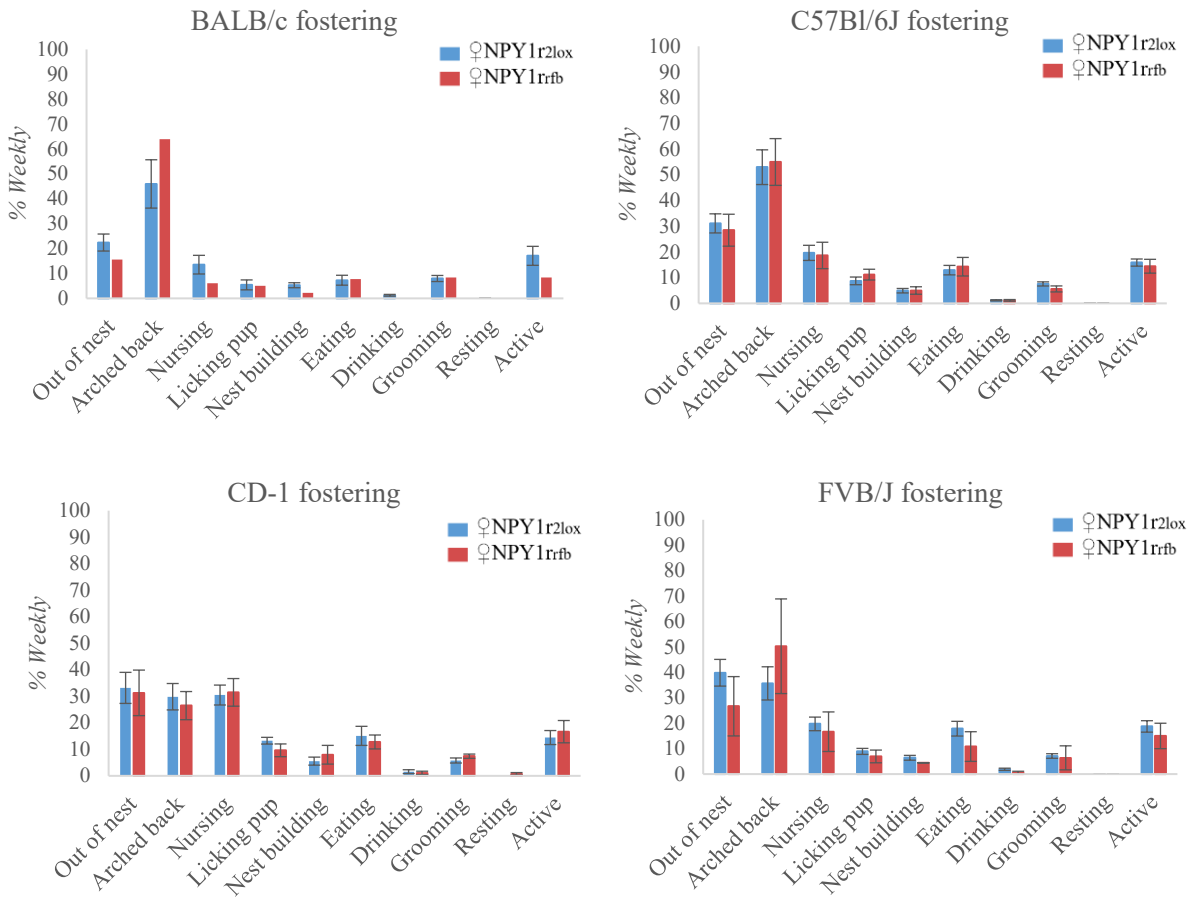


Fig. 5.19 Maternal behavior observation. No differences in maternal behavior were recorded in NPY1r^{rfb} and NPY1r^{2lox} dams. Values are presented as mean ± SEM. *indicates a significant effect with p<0.05.

5.3.6 Retrieving test

We performed retrieving test on PND 4 of pups from Balb/c, C57Bl/6J and FVB/J fostered NPY1r^{2lox} and NPY1r^{rfb} dams. Statistical analysis revealed a significant effect of genotype ($F_{(1,44)}$=5,4316; p=0.02442) on first pup retrieving latency, with NPY1r^{rfb} mothers that showed an higher amount of latency time to retrieve the first pup to the nest compared to NPY1r^{2lox} mothers. No significant statistics differences were recorded about the last retrieving of pups to the nest, and in number of pups retrieved.

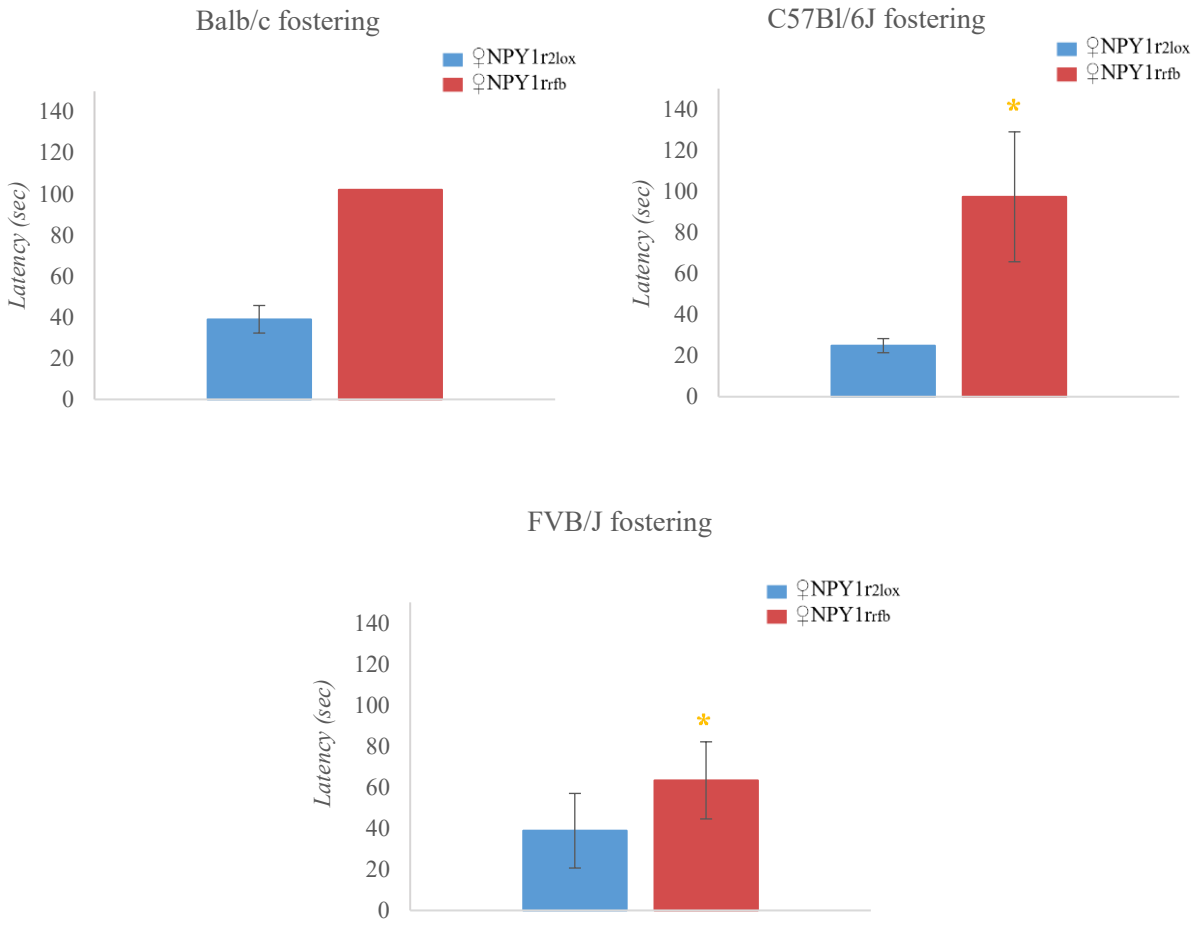
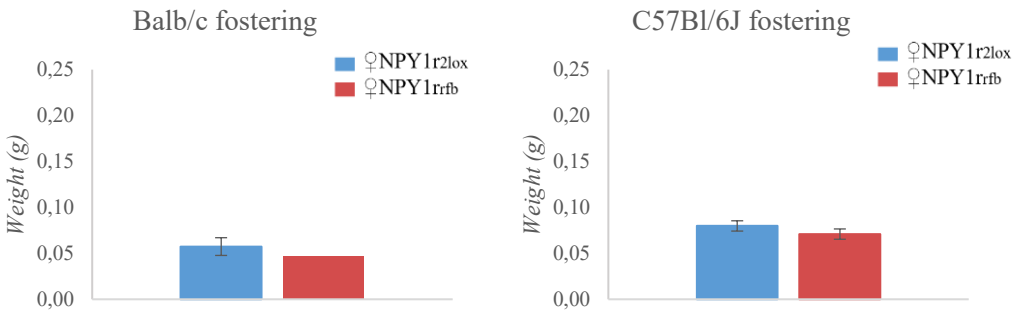


Fig. 5.20 Retrieving test. NPY1r^{rfb} dams showed higher latency of first pup retrieving compared to NPY1r^{2lox} dams. Values are presented as mean ± SEM. *indicates a significant effect with $p<0.05$. *indicates a significant effect with $p<0.05$.

5.3.7 Tissue collection

At the end of experimental procedures, samples of female's adipose tissue: brown (BAT), white (WAT) and subcutaneous (SC) fat were collected and weighed. Statistical analysis showed no differences between genotypes or strain of foster mothers.



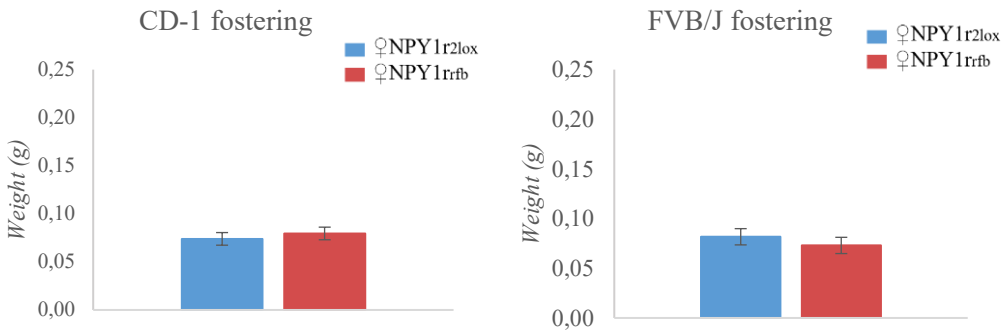


Fig. 5.21 Brown adipose tissue harvested at sacrifice. Amount of BAT in NPY1r^{2lox} and NPY1r^{rfb} female mice reared by HABN and LABN maternal care dams (Balb/c, C57Bl/6J, CD-1 and FVB/J dams). No differences were recoded. All values are presented as mean ± SEM.

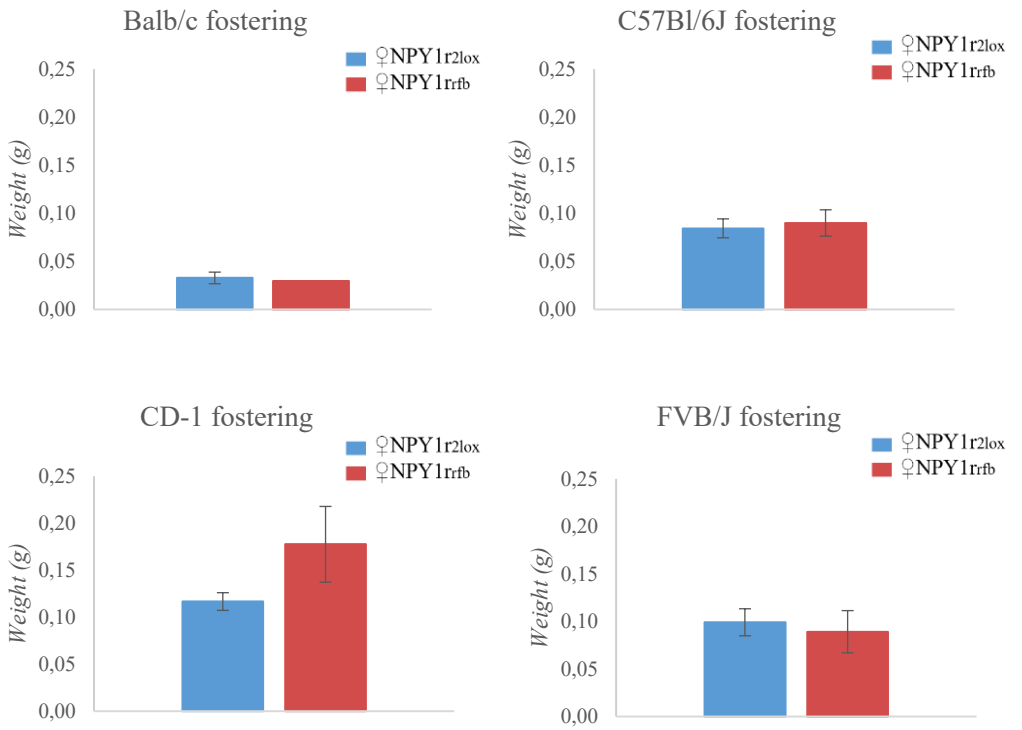
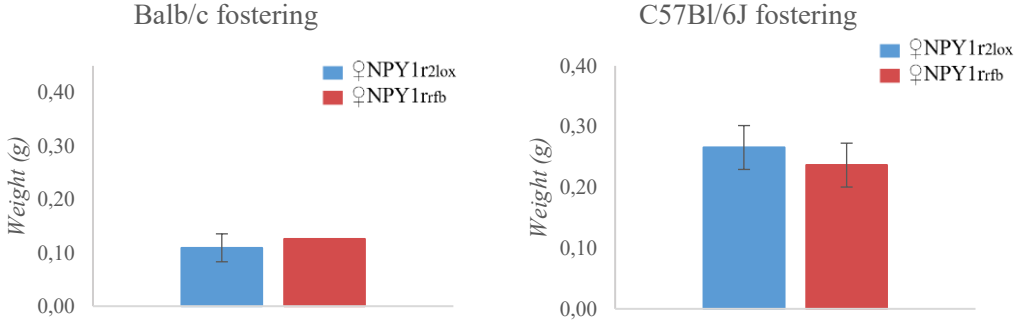


Fig. 5.22 White adipose tissue harvested at sacrifice. Amount of WAT in NPY1r^{2lox} and NPY1r^{rfb} female mice reared by HABN and LABN maternal care dams (Balb/c, C57Bl/6J, CD-1 and FVB/J dams). No differences were recoded. All values are presented as mean ± SEM.



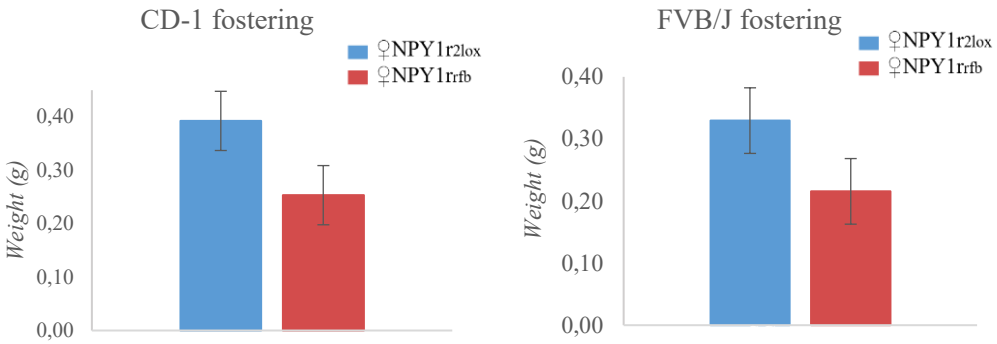


Fig. 5.23 Subcutaneous adipose tissue harvested at sacrifice. Amount of SC in NPY1r^{2lox} and NPY1r^{rfb} female mice reared by HABN and LABN maternal care dams (Balb/c, C57Bl/6J, CD-1 and FVB/J dams). No differences were recoded. All values are presented as mean ± SEM.

5.4 Discussion

In this experiment we examined the effects of limbic conditional inactivation of NPY-1r and the early maternal environment on female's behavior and reproduction.

In this study we used NPY1r^{2lox} and NPY1r^{rfb} females reared at birth by different strains of foster mothers with different levels of maternal care. In previous chapters we demonstrate that females are not affected by conditional deletion of Y1-r gene on behavior and metabolism, regardless of genotype and adoption. Females seems to be resilient to develop alterations due to the conditional inactivation of the limbic NPY-1r in emotional behavior and metabolic functions. However, when challenged with reproduction NPY1r^{rfb} females showed alterations on behavior and reproduction compared to NPY1r^{2lox} littermates.

One of the most interesting findings is that regardless to the strain of foster dams, NPY1r^{rfb} females showed lower reproductive success compared to NPY1r^{2lox} females, that means reduced pups'survival at PND 6, and lower offspring body weight at birth, but no differences were recorded in spontaneous maternal behavior although only few NPY1r^{rfb} females had at least one pup alive at PND 6, a small number of animals to appreciate differences in maternal behavior observation.

The evaluation of reproductive parameters showed a regular cycle activity (data not shown), and no alterations due to genotype or by foster strain of mothers. No differences were found in the litter size but NPY1r^{rfb} pups showed reduced bodyweight compared to NPY1r^{2lox} pups regardless to the strain of adoptions of dams. The reason for the difference in body weight of pups is not known, one of the hypotheses should be in nutritional state of the mothers, or in a genetic predisposition to intrauterine growth restriction, or in epigenetic mechanisms that can influence fetal growth (Cetin et al., 2013). Another possible explanation should be an alteration of fetal nutrition due to a reduction in placental transport by NPY1r^{rfb} females (Lager & Powell, 2012) or a difference in pups lactation post-partum immediately. The lower body weight of NPY1r^{rfb} offspring could be due to several different reasons, such as alterations in milk production and/or ejection, regulated by an epigenetic mechanism of "lactational imprinting" and by the intensity of suckling stimulus of pups (Wall & McFadden, 2012) or olfactory alterations that induce the reduction of the ability of dams to recognize pups and to develop maternal and nurturing behavior (Ehret & Buckenmaier, 1994). During the reproductive challenge, in post-partum period, we've observed that NPY1r^{2lox} spent most time crouched over the litter in the nest to nurse and to furnish heath to pups, conversely NPY1r^{rfb} females were often out of the nest, active in the cage, and their pups were scattered around the cage, without milk in their stomach. An interesting finding, in 2009, by Ladyman and Woodside demonstrated that in rats, administration

during lactation, in 3rd ventricle of an antisense oligodeoxynucleotide (ODN) targeted to NPY-Y5 receptor induce a decrease in food intake of mothers, and in litter body weight of treated females compared to controls. The Y1 and Y5 receptors were co-localized in hypothalamic brain regions and together with Y1 receptor exert a modulation on food intake, and on anxiety behavior; thus it is conceivable that there is a strong link between the regulation of energy balance and its effects on reproduction. Interestingly, Ladyman and Woodside observed no differences in the timing of milk ejections, but dams treated with Y5 antisense ODN spent significantly lower amounts of time on the nest nursing their pups. These data suggest that there's no difference in milk production by the mothers, but in total amount of milk received by pups (Sharon R. Ladyman & Woodside, 2009).

About the decreased reproductive success observed in NPY1r^{rfb} females, it's not clear the mechanism responsible for the higher mortality rate of pups. We hypothesized that the mortality of pups of NPY1r^{rfb} females is linked to an alteration in the social recognition of mothers toward pups. This hypothesis could be supported by the higher amount of latency time of knockout females in the retrieving test, and by our observation during experiments of a lack of maternal care displayed by the mothers. An increased pup mortality rate was observed also in other murine models. For example an experiment conducted on knockout melanin-concentrating hormone (MHC) knockout mouse model showed decreased survival rate of pups, higher retrieval latency, and a reduction in milk production compared to control mice (Alachkar et al., 2016). Melanin-concentrating hormone together with NPY is an orexigenic peptide involved in regulation of food intake, emotional behavior, energy homeostasis (Chung et al., 2011), and exert its effect also through Y1 receptor (Chaffer & Morris, 2002).

Taken together these data confirm the strong link between energy balance and reproductive functions. Another interesting experiment used an autism mouse model, in which was observed a high mortality rate of pups, due to the inability of mothers to establish an adequate mother-pup bonding. Pups were found scattered around the cage, neglected by mother, and without milk in their stomach. Furthermore, mothers showed a reduction in number of pups retrieved in the nest during pup retrieval assay and failed in pup-related behavior compared to controls. Lacking in maternal care of pups and increasing in offspring mortality were not related to cognitive and olfactory deficits, or hormonal abnormalities, nor in alteration in milk production (Grabrucker et al., 2021).

The failed mother-pup bonding that we observed, could be also by alterations in NPY1r^{rfb} pups. Infact, NPY^{rfb} pups seems to be inactive and less capable to stimulate dam to elicit nursing response, that implies a higher mortality. However, we need further investigation to understand

the mechanisms through which the conditional deletion of NPY-1r induce decreased body weight and reduced reproductive success in NPY1r^{rfb} females.

The second aim of the experiment was to assess the effects of pregnancy on behavior of NPY1r2^{lox} and NPY1r^{rfb} females. We perform open field test two times: before pregnancy (baseline) and during pregnancy, in line with results of elevated plus maze test in experiment 1, both the genotype showed high levels of anxiety-like behavior, with a higher amount of time spent in border zone compared to time spent in medium and center zone. NPY1r^{rfb} females reared by CD-1 dams spent less time on center zone of the arena compared to NPY1r^{2lox} females. And pregnancy induce a reduction in time spent on the center zone compared to the baseline. Females of both genotypes reared by C57Bl/6J spent more time in the medium zone during pregnancy compared to the baseline. Pregnancy has no effects on anxiety-like behavior, and as expected this physiological state induce a reduction in locomotor activity within the arena, in order to reduce energy expenditure to facilitate a positive energy balance (Ladyman et al., 2020). When tested for depressive-like behavior NPY1r^{rfb} females reared by C57Bl/6J and FVB/J foster mothers showed a lower consumption of sucrose solution compared to NPY1r^{2lox} dams, while no differences due to genotype were recorded in females reared by Balb/c and CD-1 foster mothers. In addition to anxiety-like and depressive-like behavior, we exposed females to behavioral test during pregnancy and post-partum to evaluate the maternal motivation behavior of NPY1r^{2lox} and NPY1r^{rfb} dams. In nest building assessment test, contrary as expected, no differences were observed due to genotype in females reared by CD-1 and FVB/J in the percentage of dams that made a nest with a score ≥ 4, while in females reared by Balb/c and C57Bl/6J we observed a higher percentage of NPY1r^{rfb} compared to NPY1r^{2lox} with a score ≥ 4 although in Balb/c adoption this difference not reached statistical significance.

Interestingly, when we performed retrieving test, we observed a significant difference in latency to retrieve the first pup in the nest: NPY1r^{2lox} showed lower latency time compared to NPY1r^{rfb} dams. These results showed that conditional deletion of limbic NPY-1r during the reproductive challenge affects only partially the behavioral profile of females in anxiety-like and depressive-like behavior but exerts an effect in retrieving pups by mothers. Conversely, reduced expression of NPY-1r effects were recorded in offspring features: NPY1r^{rfb} females regardless to adoption strain showed lower body weight of pups at birth, and a decreased reproductive success compared to NPY1r^{2lox} females.

However, further studies are needed to better understand the mechanism beyond conditional knockout NPY1r^{rfb} mother-pup interaction and how NPY and NPY-1r might influence this interaction.

Chapter 6

6.1 Experiment 3: Effects of cross fostering procedure on reproductive success of NPY^{2lox} and NPY1r^{rfb} females reared by CD-1 foster mothers

In this experiment we used NPY^{2lox} and NPY1r^{rfb} females reared by CD-1 foster mothers at birth. The timeline of the experiment was the same described in chapter 5 for the Experiment 2. The purpose of this experiment was to assess if cross fostering procedure can exert an effect on reproductive success. At PND 143 females were mating with C57BL/6J wild-type males. After one week, males were removed from female's cages. On the day of delivery, pups were sexed and weighed as well, then cross fostering was performed, and experimental group were created as follow:

Not cross-fostered:

- NPY^{2lox} females left undisturbed with their pups (n=15)
- NPY1r^{rfb} females left undisturbed with their pups (n=15)

Cross-fostered:

- NPY^{2lox} cross fostered with NPY1r^{rfb} (n=8)
- NPY1r^{rfb} cross fostered with NPY^{2lox} (n=8)

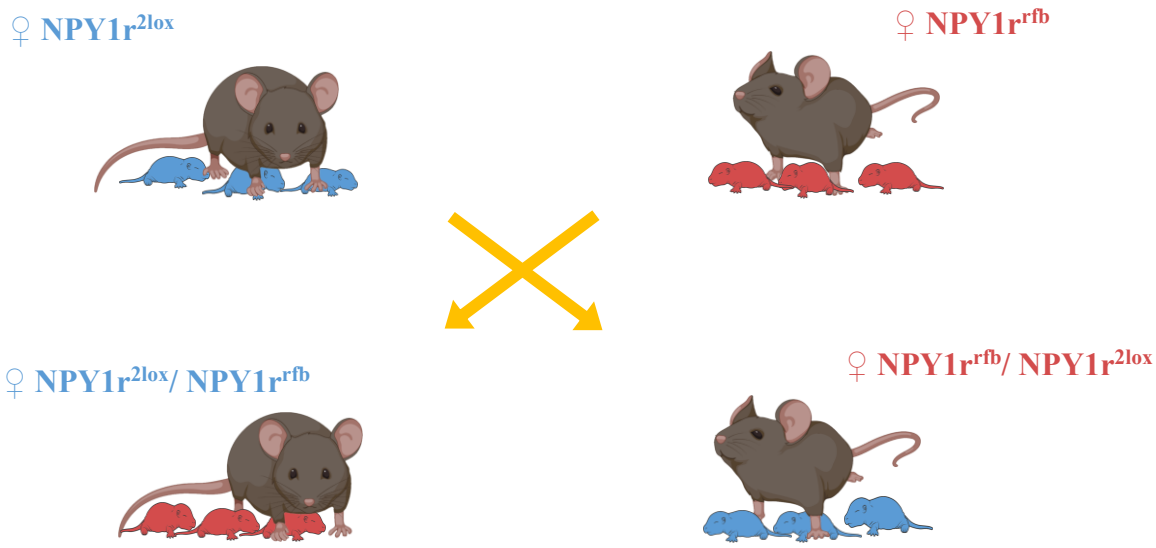


Fig. 6.1 Cross-fostering procedure scheme. At the top in the picture NPY1r^{2lox} and NPY1r^{rfb} females left indisturbed with their own pups. In the bottom of picture NPY1r^{2lox} mothers cross-fostered with NPY1r^{rfb} pups, and NPY1r^{rfb} female cross-fostered with NPY1r^{2lox} pups. *(Created with BioRender.com)*

6.2 Materials and methods

6.2.1 Open field test (OF)

To assess anxiety-like behavior we performed OF test. Animals were placed in the test room at least 3 hours before the beginning of the test (habituation period to new environment and different light). Each animal was placed in a corner of the open arena of polyethylene (53cm x53cm) and left free to explore them for 5 minutes. The arena surface was divided in 3 concentric zones: border, medium and center. Each session of test was videotaped with a camera placed above the arena. All the trials of test were analyzed with an ethological software system (The Ethovision, Noldus, NL). For each subject tested we scored: time spent in each zone of the arena and the total distance traveled by the animals. To evaluate anxiety level of animals we consider time spent in the center zone. We performed OF test two times: before reproductive event (baseline), and during pregnancy. Between trials arena was cleaned 3 times: with water, with alcohol and finally water.

6.2.2 Sucrose preference test

Sucrose preference test was used to evaluate anhedonia, the decreased response to rewards, that is associated to depressive state. To evaluate depressive-like behavior during reproductive event, experimental animals were subject to sucrose preference test. Each animal can choose between two bottles placed in their home cage: one of the bottles contain water solution, and the other contain a 1% sucrose solution. All bottles were weighed two times: before the beginning of the test, and 24 hours later. Sucrose preference was expressed as percentage of sucrose solution consumed on the total amount of liquid intake by every experimental animal.

6.2.3 Nest building assessment test

At PND 160, the day before the expected delivery, we were evaluated nest building of females, as a measure of maternal motivation. The test consists in 3 observations, for 6 hours. At 9:00 AM a pressed cotton square (5cm x 5cm, “Nestlet”, Datesand Ltd.) was placed in homecage of every female. Nests were assessed at 11:00 AM, 1:00 PM and 3:00 PM, and we used a rating scale from 1 to 5 to score the nest (Deacon, 2006). Values of rating scale are the following:

- **1** Nestlet not noticeably touched (> 90 % intact)
- **2** Nestlet partially torn up (50-90 % intact)
- **3** Nestlet mostly shredded (> 50% torn up)

- **4** Nestlet torn up, material is gathered into a nest, with walls less than mouse body height (curled up on its side) on 50 % of its circumference.
- **5** Nestlet is totally torn up, with walls larger than mouse body height (curled up on side) on 50 % of its circumference.

6.2.4 Delivery and body weight pups

We recorded the number of the days from mating (the day in wich male was placed into female's cage) to delivery. Offspring body weight growth was monitored at birth (PND 0) and at PND 6 with a digital balance accurate to 0.01 g (Sartorius, Germany). We recorded also the number of pups in each litter, and reproductive success, defined as the percentage of dams with at least one pup alive at PND 6. We also monitoring body weight of females four time during the reproductive challenge: the day of mating, when male were removed from female's cage, at delivery and at sacrifice.

6.2.5 Maternal behavior observation

During the first week of pup's life, we observed spontaneous maternal behavior of knock-out and control females. Maternal behavior observation procedure was previous described in "Foster mothers maternal behavior observation" paragraph.

6.2.6 Tissue collection

Females were sacrificed by decapitation at PND 6 of pups, following CO2 exposure. Trunk blood was collected in heparinized tubes (Sarsted) and centrifuge 4000 RPM at 4°C for 10 minutes. Brain was collected and frozen in liquid nitrogen for later analysis.

6.2.7 Statistical analysis

A 2-way ANOVA (genotype and adoption) was run to analyze open field test, sucrose preference test, retrieving test, offspring body weight at birth and litter size and spontaneous maternal behavior. When necessary, a post-hoc analysis through Tukey Test was performed. Reproductive success was analyzed by means of the Fisher-exact p test. A Wilcoxon matched pairs test was used to analyzed days passed from mating to delivery. Reproductive success was analyzed by means of the Fisher-exact p test. Data were analysed with Statistica 10.0 software (Stat-Soft,

Tulsa, OK, USA). Significance was determined when $p<0.05$. Data are presented as mean ± standard error of the mean.

6.3 Results

6.3.1 Open field test

NPY1r^{2lox} and NPY1r^{rfb} female mice were tested for anxiety-like behavior through the OF test before and during pregnancy. Both during baseline and pregnancy, NPY1r^{rfb} female mice showed a trend to spend most of the time in the borders of the arena ($F_{(1,11)}=3.9367$; $p=0.07276$) and less time in the medium zone ($F_{(1,11)}=3.8408$; $p=0.07584$) compared to NPY1r^{2lox}. A significant reduction of time spent in the center zone was observed in pregnant NPY1r^{2lox} and NPY1r^{rfb} females as compared to the baseline test ($F_{(1,11)}=12,416$; $p=0.00477$). Furthermore, regardless of genotype, pregnant NPY1r^{2lox} and NPY1r^{rfb} females showed significantly reduced locomotor activity (distance moved in the arena) compared to the baseline ($F_{(1,11)}=96,114$; $p<0.01$.

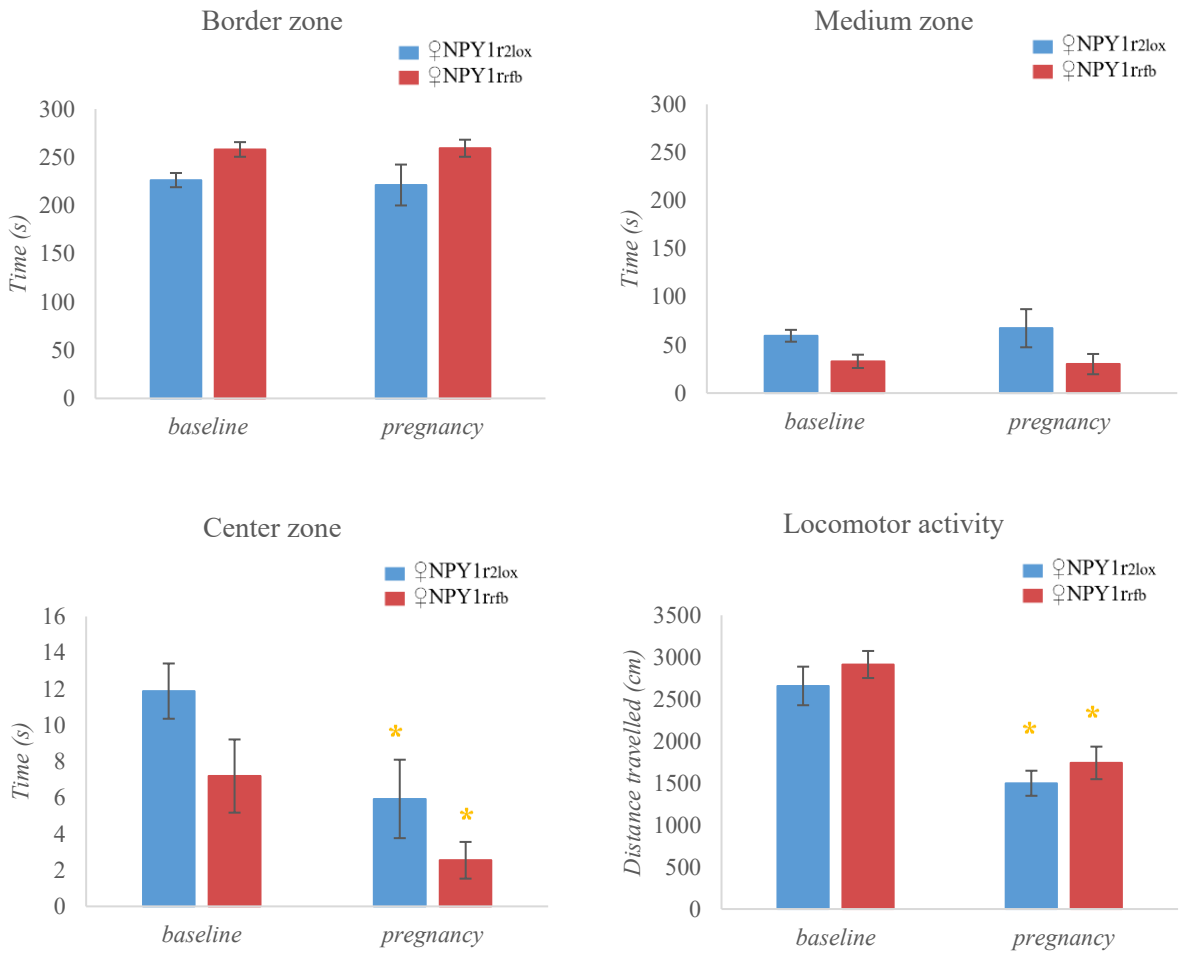


Fig. 6.3.1 Open field test. a) Time spent in the border, **b)** medium and **c)** center zone of the arena and **d)** total locomotor activity. NPY1r^{rfb} females showed a trend to spend more time in the border zone than in medium zone. During pregnancy females spent

less time in the center zone regardless to genotype. Total locomotor activity decreases during pregnancy in both genotypes. Values are presented as mean ± SEM.

6.3.2 Sucrose preference test

As pictured in Fig. 6.2, no differences were observed in depressive-like behavior due to genotype between NPY1r^{2lox} and NPY1r^{rfb} females.

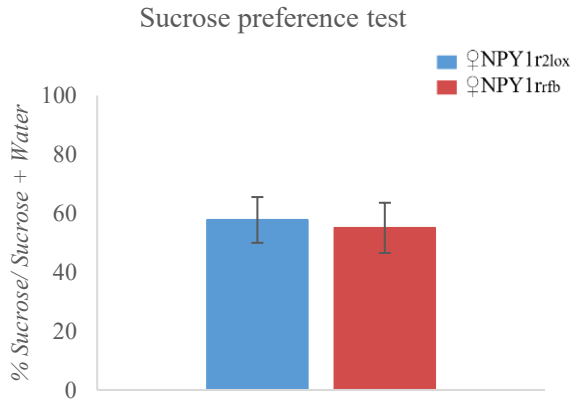


Fig. 6.2 Sucrose preference test. No differences were recorded in depressive-like behavior of females of both genotypes. Values are presented as mean ± SEM.

6.3.3 Nest building assessment

After two hours from the placement of the nestlet, 50% of NPY1r^{2lox} and 60% of NPY1r^{rfb} pregnant females showed a nest score ≥ 4. At the last observation (6 hours), 62,50% of NPY1r^{2lox} pregnant female showed a perfect nest whereas 80% of NPY1r^{rfb} pregnant females built a perfect nest (Fig.6.3). Statistical analysis showed no differences between genotype in nest building.

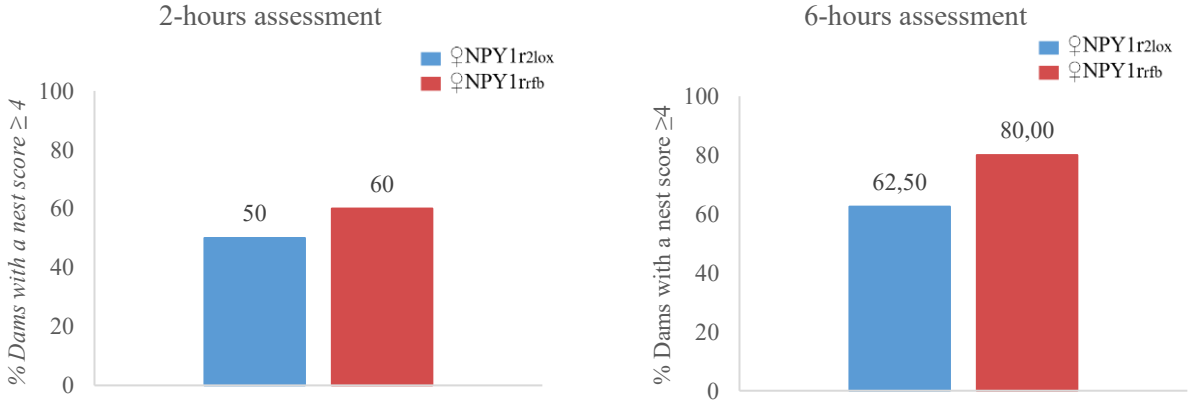


Fig. 6.3 Nest building assessment. 2 hours and 6 hours assessment, data showed as percentage of dams with score ≥ 4. No differences were recorded due to genotype.

6.3.4 Reproductive event

Days from mating to delivery

Days from the mating to the delivery of NPY1r^{2lox} and NPY1r^{rfb} female mice were recorded. We defined "day of mating" the day in which male was placed in the females'cage and counted the days from "day of mating" to the exact day in which females gave birth (PND 0). A Wilcoxon matched pairs test showed that NPY1r^{rfb} gave birth after a lower number of days from mating to P0 than NPY1r^{2lox} females (z=5,905164, p<0.001).

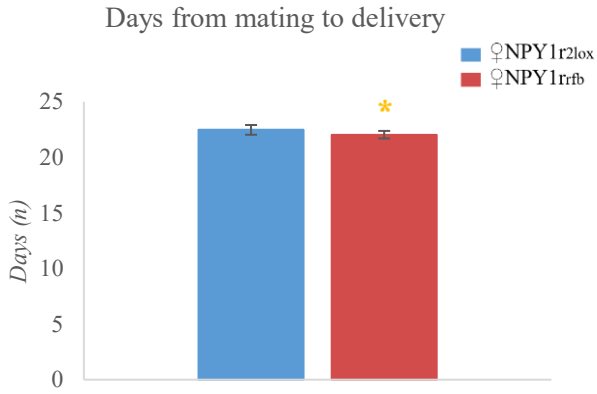


Fig. 6.3.4 Days from mate to delivery. NPY1r^{rfb} females showed a lower number of days from mate to delivery compared to NPY1r^{2lox} females. Values are presented as mean ± SEM.

Body weight of pups

Offspring of NPY1r^{2lox} and NPY1r^{rfb} females was sexed and weighed within 12 hours after birth (PND 0). Although no differences were observed in the number of pups delivered, as showed in pictures, NPY1r^{rfb} pups showed significant lower body weight compared to NPY1r^{2lox} pups ($F_{(1,44)}$=5,1773; p=0.02781).

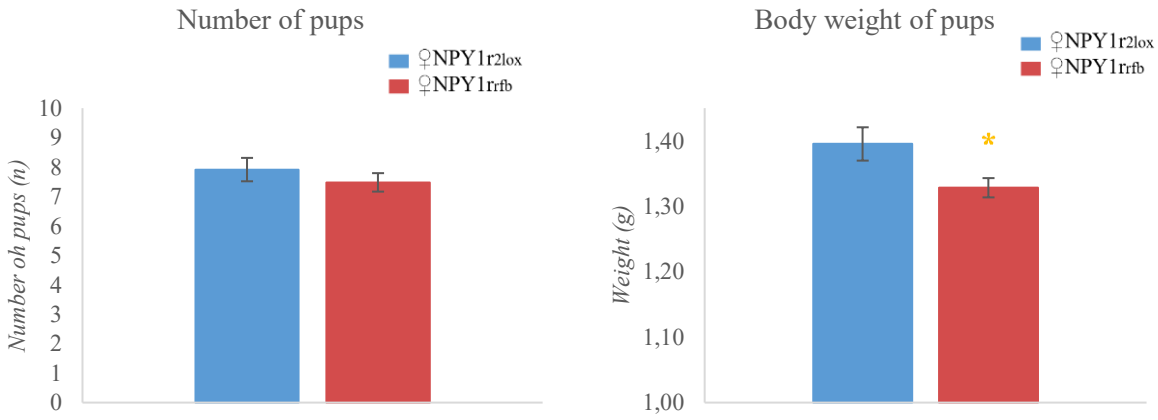


Fig. 6.3.5 Number and body weight of pups. No differences in number of pups delivered were observed in both genotypes. Pups from NPY1r^{rfb} females showed significant lower body weight at birth compared to NPY1r^{2lox} pups. Values are presented as mean ± SEM. *indicates significant difference with p<0.05.

Body weight during

Body weight was monitored during the main steps of reproductive challenge: mating, male separation, delivery and sacrifice. Monitoring of body weight from mating to the delivery was performed on pregnant females whereas analysis of body weight from the delivery to sacrifice was performed only on females presenting at least one pup alive on PND 6. As showed in Fig. 6.6 A, no differences in body weight were observed from mating to the delivery due to genotype. At birth of pups, four experimental groups were created (not cross-fostered NPY1r^{2lox} and NPY1r^{rfb} and cross-fostered NPY1r^{2lox} and NPY1r^{rfb} females). Analysis of body weight from the delivery to sacrifice showed a difference between non-cross-fostered and cross-fostered dams (Fig.6.6 B). NPY1r^{rfb} and NPY1r^{2lox} females showed a greater increase in body weight from the delivery to sacrifice as compared to cross-fostered NPY1r^{2lox} and NPY1r^{rfb} females (effect of activity: $F_{(1,13)}$=23,215; p=0.00034).

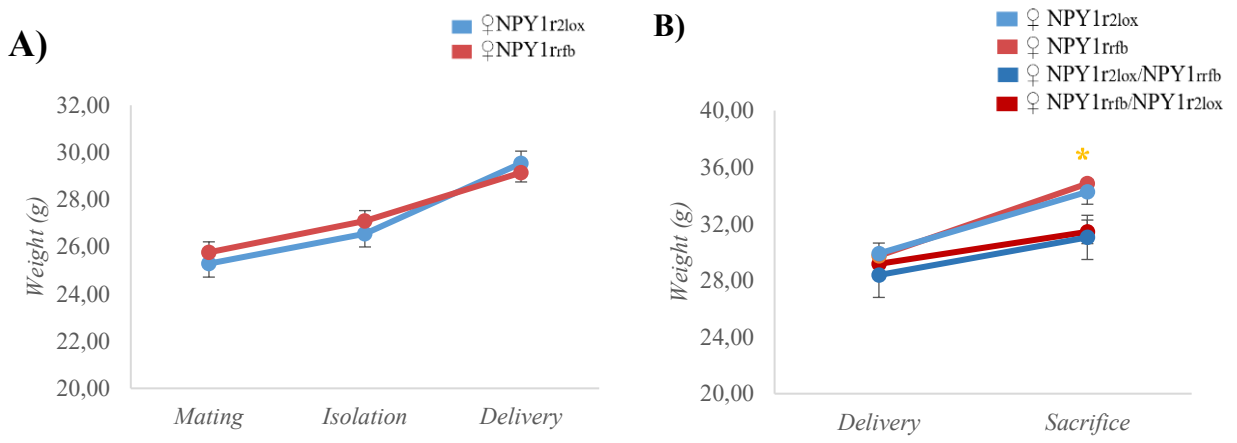


Fig. 6.6 Body weight during reproductive event. No differences were observed in both genotypes and strain of adoption. Values are presented as mean ± SEM. *indicates significant difference with p<0.001.

Reproductive success

We analysed reproductive success, defined as the number of NPY1r^{2lox} and NPY1r^{rfb} females who had at least one pup alive 7 days after giving birth (PND 7). As pictured in Fig.6.7, only 20% of NPY1r^{rfb} females left with their pups showed at least one pup alive, on the contrary 80% of NPY1r^{2lox} females showed at least one pup alive at P6 (Fisher exact p, one-tailed p<0.001). As showed in picture, cross-fostering procedure influenced the reproductive success in both genotypes, in fact, NPY1r^{2lox} females showed a reduction in reproductive success (from 80% to

50%), in contrast to NPY1r^{rfb} females that show an increased in reproductive success (from 20% to 50%).

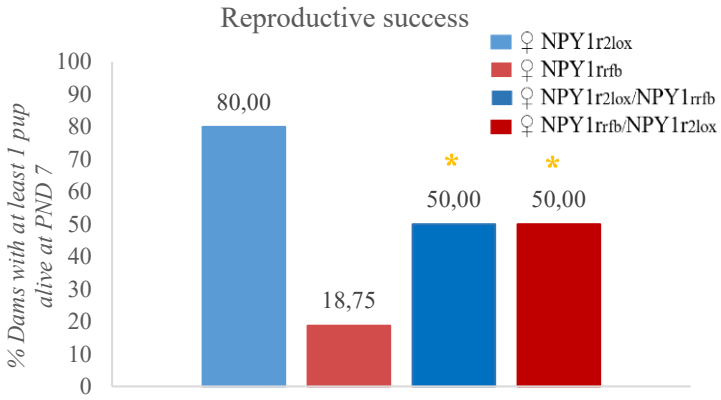


Fig 6.7 Reproductive success. NPY1r^{rfb} showed lower reproductive success compared to NPY1r^{2lox}females. Cross-fostering procedure induce an increasing in NPY1r^{rfb} and a decreasing in NPY1r^{2lox} reproductive success. We define reproductive success as the % of the dams with at least one pup alive at PND 6. *indicates a significative difference with p<0.001.

6.3.5 Maternal behavior observation

We performed maternal behavior observation during the first week of pup's life. The protocol was the same described in chapter 3. Statistical analysis showed no differences in pup-related and not pup-related behaviors between the four groups considered: NPY1r^{2lox}, NPY1r^{rfb}, and NPY^{2lox} cross-fostered with NPY1r^{rfb} and viceversa, NPY1r^{rfb} cross-fostered with NPY1r^{2lox} dams.

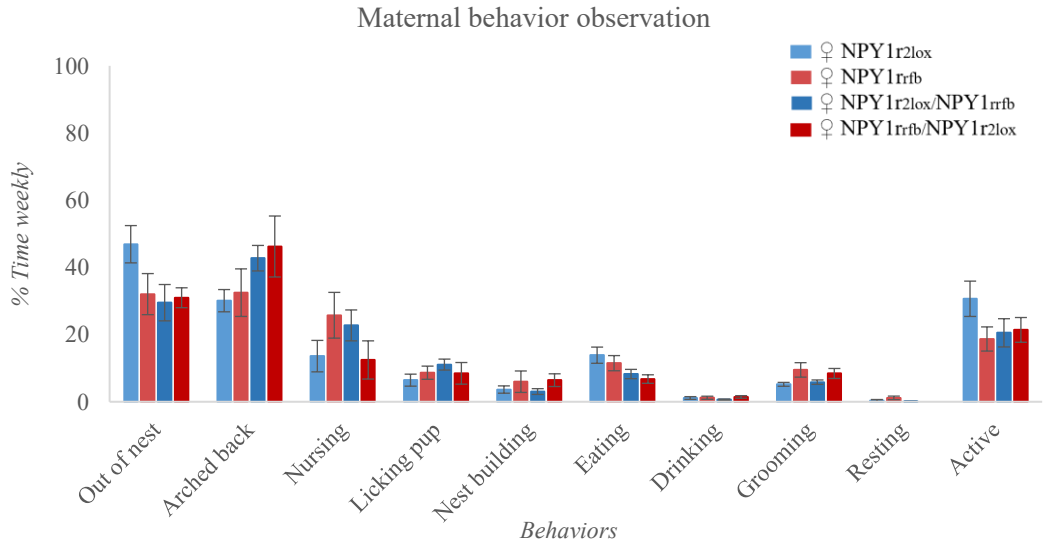


Fig 6.8 Maternal behavior observation. Maternal behavior observation from PND 1 to PND 7, of NPY1r^{rfb} and NPY1r^{2lox} females and NPY1r^{rfb} and NPY1r^{2lox} cross-fostered. No differences were recorded. Values are presented as mean ± SEM.

6.4. Discussion

In this experiment we focused on NPY1r^{2lox} and NPY1r^{rfb} females reared by CD-1 strain of foster mothers characterized by high quality of maternal care. The aim of this experiment was to assess if cross-fostering procedure between NPY1r^{2lox} and NPY1r^{rfb} mice might affect the reproductive success in females.

At this aim, we used four experimental groups: 1) NPY1r^{2lox} left with their own pups; 2) NPY1r^{rfb} left with their own pups; 3) NPY1r^{2lox} cross-fostered with NPY1r^{rfb} 4) NPY1r^{rfb} cross-fostered with NPY1r^{2lox}. When we evaluate reproductive success, we observed the 80% of NPY1r^{2lox} with at least one pup alive at PND 7, while only the 19% of NPY1r^{rfb} mice had at least one pup alive at PND 7.

Interestingly, the cross-fostering procedure influenced reproductive success in both the genotype. In fact, NPY1r^{2lox} showed a decrease in the percentage of reproductive success (80% vs 50%), conversely NPY1r^{rfb} females showed an increase of reproductive success (19% vs 50%). These data suggest that we could hypothesized the contribution of a combination of factors in the correct interaction between mothers and pups.

The observation of spontaneous maternal behavior showed no significative difference in time spent in pup-related and non-pup-related by the four experimental groups of dams. Analysis of reproductive parameters showed a shorter time from mating to delivery in NPY1r^{rfb} females compared to controls. However, no difference was recorded for litter size, but NPY1r^{rfb} offspring showed a lower body weight at birth, as discuss in previous chapter.

Monitoring of body weight of dams at three different time (mating, isolation and delivery) showed no difference due to genotype, body weight recorded at delivery and at sacrifice, in cross-fostered and non cross-fostered NPY1r^{2lox} and NPY1r^{rfb} females, showed a higher body weight at sacrifice compared to non cross-fostered dams of both genotypes. During pregnancy NPY1r^{2lox} and NPY1r^{rfb} females were tested for emotional behavior. Anxiety-like behavior was assessed through the Open Field Test (OPF) performed before mating (baseline) and during pregnancy. Results showed an increase in anxiety levels, during pregnancy, regardless of genotype, females spent less time in the center of arena, and decreased locomotor activity compared to the baseline. No differences were recorded in depressive-like behavior and in nest building assessment due to genotype.

The results of this experiment confirm what we observed in reproductive challenge described in Chapter 5. The conditional deletion of limbic NPY-1r affect anxiety-like behavior during pregnancy, but not depressive-like behavior in CD-1 reared females of both genotypes. Maternal motivation behavior evaluated through nest building assessment showed no differences between

NPY1r^{2lox} and NPY1r^{rfb} females. The most relevant findings were the effect of cross-fostering on reproductive success, when cross-fostered NPY1r^{2lox} females showed decreased survival rate of pups conversely to NPY1r^{rfb} females that showed an increased in pups' survival. However, further analysis are needed to explain the underlying reasons of the unsuccessful mother-pup interaction observed in NPY1r^{rfb} females.

Chapter 7

Brain analysis (still in progress)

During pregnancy mammalian female brain is characterized by structural and functional modification that involves different areas and neuronal circuitries. These changes begin during gestation and persists throughout early post-natal period and lactation, depending on the demands of pups (Hillerer et al., 2014). Therefore neuronal plasticity of the dam's brain it's required to ensure an adequate response to pups requirements (Russell et al., 2001). The medial preoptic area (mPOA) is one of the most important brain area involved in the expression of maternal behavior and social bonding behavior in rodents (Numan & Stolzenberg, 2009). Lesions, especially in the dorsal mPOA induce the inhibitions of retrieving without affecting feeding, locomotion or sexual behaviors in females (Jacobson et al., 1980). The mPOA area is necessary to the onset and early expression of maternal behavior via reception of pup-related information from sensory modalities and it's responsible to promote responsiveness to pup-related stimuli at parturition (riferimenti). During the reproductive cycle morphology of MPOA neurons undergoes changes in spine density, number and length of basal dendritic branches and an increase in somatic size (Frankfurt et al., 2011; Keyser-Marcus et al., 2001).

Regulation of neural plasticity involves the construction of the stable extracellular matrix (ECM) across peculiar structure called perineuronal nets (PNNs). Perineuronal nets are lattice-like aggregates of multiple molecules including chondroitin sulfate proteoglycans (CSPGs), hyaluronan and glycoproteins and they're important to the formation of stable neural circuits in adult brain contributing to the closure of developmental of critical periods (Song & Dityatev, 2018). PNNs surround both excitatory and inhibitory neurons, in several brain areas PNNs ensheath GABA-ergic parvalbumin-expressing neurons (Burket et al., 2021).

In recent years, researcher attention was focused on potential role of PNNs in CNS. Much of the knowledge of the effects of CSPGs and PNNs stems from their modification by digestion of the GAG chains using chondroitinase ABC (ChABC). The degradation of PNNs by ChABC in basolateral amygdala (BLA) medial prefrontal cortex (mPFC), anterior cingulate cortex, BLA, or auditory cortex impairs the expression of fear conditioning in rodent models (Gogolla et al., 2009). Furthermore it was demonstrated the implication of PNNs in spontaneous object recognition memory. The attenuation of PNNs in hippocampus and perirhinal cortex can increase

synaptic transmission and facilitate long-term depression (LTD) in the perirhinal cortex or CA1 region, and this correlates with enhanced recognition memory (Khoo et al., 2019; Romberg et al., 2013).

Beyond the object recognition memory, it was demonstrated that the regulation of PNNs in spatial memory processing. In the conditional knock-out mouse model for NPY-1r used in this study, it was demonstrated that overexpression of PNNs in hippocampal CA1 area affects spatial memory learning in $NPY1r^{rfb}$ mice (Bertocchi et al., 2021).

Although (especially in recent years) the knowledge about the role of PNNs made progress, little is known regard the implications in brain functions during pregnancy and in post-partum period. Uriarte e colleagues demonstrated that in rats that the expression of PNNs in mPOA increase during gestational period substained by gonadal hormones action, following by a minimum peak at delivery and a subsequent increase in the first week of pup's life, with a subsequent final reduction at the end of lactation period (Uriarte et al., 2020). The complexity in PNNs trend during pregnancy and lactation suggests a fine regulation of neural circuits mediated by PNNs. Fluctuability in PNNs levels observed in this experiment could lead to the peculiar state of pregnancy, characterized by dynamically changes adjusting to the hormonal environment and to the physiological needs of pups.

Aimed to investigate any differences in the PNNs expression that could lead to the different reproductive success rate and to what we observed in behavioral experiment discuss in previous chapters, we performed the analyses of PNNs of $NPY1r^{2lox}$ and $NPY1r^{rfb}$ females reared by CD-1 dams. Analyses are still in progress.

Chapter 8

General discussion and conclusions

The phenotype is the set of morphological and functional traits of an organism, resulting from the interaction between genotype and environmental factors. In mammals, early maternal environment plays a key role in the regulation of postnatal phenotypic development. Several studies in rodent models showed the influence of maternal cares on behavior, stress response and metabolism in adult offspring. In the mouse model used in this work, characterized by a conditional deletion of Y1 receptor of neuropeptide Y (NPY) gene in excitatory neurons of the forebrain, the gene deletion influences the phenotype, in relation to the early maternal environment.

NPY is a neuropeptide involved in several different physiologic functions, among wich regulation of daily food intake and regulation of energy balance (Eva et al., 2006); stress response and ethanol intake (Heilig & Thorsell, 2002), hormonal secretion (LM et al., 1994) emotional behavior (Domschke et al., 2010) and reproduction (Satya P. Kalra & Kalra, 2004). NPY exert these functions through Y1 G-protein coupled receptor largely expressed in the limbic area which hippocampus, amygdala, bed nucleus of the stria terminalis and in the hypothalamic areas such as medial preoptic area, paraventricular nucleus, dorsomedial nucleus, ventromedial nucleus, arcuate nucleus (Eva et al., 2006).

The conditional deletion restricted to the excitatory neurons of the forebrain in murine model used in this study has highlighted the role of limbic Y1r expression in regulating energy balance and anxiety-related behavior (Bertocchi et al., 2011) reduction in body weight growth and increased anxiety levels were observed when mice were reared by HABN mothers, suggesting a key role of the early maternal behavior in regulation of NPY-1r hippocampal expression (Bertocchi et al., 2011). Moreover, NPY system shows sex-dependent difference and is sensitive to gonadal steroid action that results in the development of dimorphic phenotypes (Eva et al., 2020).

NPY also acts as mediator among energy balance, gonadotropin-releasing hormone (GnRH) secretion and sexual behavior (Hill et al., 2008). Based on this evidence, we perform three experiments with a detail focus on NPY-1r effects on females. The first step of the experimental procedures was to evaluate maternal care quality of four different strains of foster mothers. Through the crossing of three different mouse lines we generate a $NPY1r^{2lox}$ and $NPY1r^{rfb}$ males

and females offspring, reared at birth, by foster mothers belonging to one of the following strains: Balb/c, C57Bl/6J, CD-1 or FVB/J dams.

The analysis of spontaneous maternal behavior in the first week of pups life confirm higher levels of maternal care displayed by CD-1 and FVB/J dams compared to Balb/c and C57Bl/6J dams. Time spent in arched back nursing (ABN) and total nursing by CD-1 and FVB/J dams (HABN mothers) is significantly higher than Balb/c and C57Bl/6J dams (LABN mothers). Monitoring of body weight from PND 34 to PND 90 showed lower body weight of $NPY1r^{rfb}$ males compared to $NPY1r^{2lox}$ reared by HABN mothers whereas no differences were recorded in females regardless to genotype or adoption, confirming what observed by Bertocchi e colleagues (Bertocchi et al., 2011).

In the first experiment we want to assess sex differences in behavioral and metabolic phenotype of $NPY1r^{2lox}$ and $NPY1r^{rfb}$ males and females, reared by four different strains of foster mothers. We examined anxiety-like, aggressive-social behavior and response to novelty. Our findings show that conditional NPY-1r inactivation induces behavioral effects only in males reared by high maternal care dams by reducing aggressive behavior. Interestingly in females reared by C57Bl/6J mothers this effect seems to be inverted and $NPY1r^{rfb}$ female mice showed a reduction of the percentage of agonistic behavior. In the Resident/Intruder paradigm $NPY1r^{rfb}$ male mice were characterized by higher attack latency when reared by high maternal care dams and consequently, they spent less time than control mice engaged in agonistic behavior.

These results are in apparent contrast to what was described by Karl et al. (2004) who said that germinal ablation of the Y1 receptor gene led to a pronounced increase in territorial aggressive behavior by affecting the serotoninergic system. Consistently, $NPY1r^{2lox}$ mice, reared by FVB/J and CD-1 dams, characterized by high expression of NPY-1r in these hippocampal areas showed higher aggressive behavior than $NPY1r^{rfb}$ mice.

However, when reared by C57Bl/6J dams the percentage of time spent by $NPY1r^{rfb}$ male mice in agonistic behavior increases, suggesting that levels of maternal care might be a key to better understand aggressive behavior in adults. As expected, levels of agonistic behaviour in females are significantly lower than males. Female mice spent most of the time in social investigation: $NPY1r^{2lox}$ females displayed more social behavior than $NPY1r^{rfb}$ females excepted for females reared by C57Bl/6J.

Anxiety-like behavior and response to novelty were assessed through EPM and NISF tests. We observed that both $NPY1r^{2lox}$ and $NPY1r^{rfb}$ males and females reared by high or low maternal care dams spent most of the time in the close arms of the EPM and rarely explored the open arm, suggesting that, regardless of sex, genotype and adoption, they are characterized by an anxious-

like phenotype and high trait-anxiety. Contrary to what expected, reduced NPY-1r mRNA expression in limbic areas did not affect anxiety-like behavior in the EPM test, but mice reared by Balb/c and FVB/J dams spent less time and a lower frequency of entrance in the open space of the apparatus. Results of NISF test showed that regardless of the adoption, both male and female NPY1r^{2lox} and NPY1r^{rfb} mice showed a regular daily reduction in the latency to consume the peanut, but the latency only increased in NPY1r^{2lox} male mice when the peanut was presented in a clean bedding-filled cage on day 4. Thus indicate that NPY1r^{rfb} males present lower anxiety-like behavior as compared with their controls: these results contrast with our observation in the EPM, where both control and conditional knockout mice displayed a behavioral profile indicative of high anxiety. NPY1r^{rfb} male mice reared by CD-1 and FVB/J dams showed significantly shorter latency to eat the peanut on all trials as compared to their controls, while NPY1r^{rfb} and NPY1r^{2lox} males reared by Balb/c and C57Bl/6J showed a similar trend. No differences due to genotype or adoption were observed in female mice.

These data indicate that, regardless the adoption, male and female NPY1r^{2lox} and NPY1r^{rfb} mice are not so sensitive to novelty-induced inhibition of feeding, as they did not show the expected environment-induced avoidance in approaching the highly palatable food. Since these effects are more pronounced in males and especially in NPY1r^{rfb} males reared by HABN mothers, this suggest that a reduction of hippocampal NPY-1r may affect brain paths involved in the processing of reward-related behavioral responses. We hypothesize that discrepancies in results of EPM and NISF tests indicates that behavior displayed by our subjects in the NISF test might be an indicator of an enhanced reward-related response. Another explanation could be the display of an autistic-like response: the high anxiety displayed in the EPM associated by insensitivity to a novel environment shown in the NISF test might be factors related to autism spectrum disorder and the absence of the inhibition of palatable food intake in novel environment might be interpreted as a resistance to change and response to the changes in routine, characteristics of autism (Crawley, 2007).

In this experiment we also evaluate the the effects of limbic NPY-1r on energy balance and vulnerability to high fat diet induced obesity and metabolic syndrome of NPY1r^{2lox} and NPY1r^{rfb} mice. Results demonstrated that regardless of the adoption, both NPY1r^{rfb} and NPY1r^{2lox} male mice fed HFD showed increased body weight growth than STD fed groups, NPY1r^{rfb} males showed much greater body weight gain than their control (NPY1r^{2lox}) when on HFD. This greater increase in body weight started from the second day of HFD and persisted throughout the 21 days of the diet regimen. Thus, contrary to what reported in males, NPY1r^{rfb} female mice did

not show lower body weight growth and when challenged with high fat diet, they didn't develop diet-induced obesity and glucose intolerance.

As expected, in male mice, HFD induced an increase of abdominal adipose tissue in both genotypes than the STD groups, regardless of the adoption. Besides, $NPY1r^{rfb}$ on HFD showed much greater WAT than $NPY1r^{2lox}$ mice on HFD only when reared by high maternal care dams (CD-1 and FVB/J). At last, $NPY1r^{rfb}$ mice on HFD also showed impaired glucose clearance in the glucose tolerance test. Even though HFD did not induce fasting hyperglycemia in either genotype, $NPY1r^{rfb}$ mice reared by high maternal care mothers on HFD showed higher blood glucose level as compared to their controls 30 minutes after the glucose injection, indicating impairment in glucose clearance. This effect is not remarkable in $NPY1r^{rfb}$ vs $NPY1r^{2lox}$ fostered at birth to C57Bl/6J or Balb/c dams.

These results suggest that $NPY1r^{rfb}$ male mice reared by HABN mothers are characterized by a phenotype that predisposes them to develop metabolic disease as demonstrated by their high abdominal adipose tissue and glucose clearance impairment both of which are predictive factors for obesity and metabolic syndrome (Björntorp, 1991b; Jensen, 2008; A H Kissebah & Krakower, 1994).

The *in situ* hybridization performed at the end of metabolic challenge on both groups (STD- and HFD-fed mice) showed that NPY-1r mRNA expression in $NPY1r^{rfb}$ mice on STD was reduced in CA1, CA3 and DG as compared to $NPY1r^{2lox}$ mice (Paterlini et al., 2021). This expression profile is similar to the one observed in male mice reared by FVB/J dams in Bertocchi 2011. After the high fat diet regimen, however, we observed an upregulation of NPY-1r mRNA expression in CA1 and CA3, in $NPY1r^{rfb}$ males fed with HFD and interestingly, the differences in the expression of NPY-1r mRNA between $NPY1r^{rfb}$ and $NPY1r^{2lox}$ disappeared.

This finding may suggest that low limbic NPY-1r expression might be responsible for the enhanced predisposition to develop diet-induced obesity and glucose intolerance (metabolic syndrome) in response to high fat diet, which in turn may induce increased expression of NPY-1r mRNA in limbic structures due to compensatory mechanisms. Opposite to what observed in male mice $NPY1r^{rfb}$ females seemed to be "shielded" from the effect of a hyper caloric food. When challenged with a HFD, both $NPY1r^{rfb}$ and $NPY1r^{2lox}$ female mice, regardless of the adoption, only showed a slight increase in body weight and a small gain of abdominal adipose tissue as compared to the control group on STD diet.

Interestingly, these effects are found regardless of genotype and adoption, suggesting that neither lower expression of NPY-1r in limbic areas and the quality of maternal care are factors promoting metabolic disorders in females. Furthermore, $NPY1r^{rfb}$ and $NPY1r^{2lox}$ females on

either STD or HFD diet showed similar blood glucose level curves after glucose administration. These weaker effects of high fat diet on females may be due to several factors and environmental variables. First of all estrogens have been shown to act like “protectors” against metabolic effects of high fat diet (Litwak et al., 2014; Riant et al., 2009).

Conditional deletion of NPY-1r in forebrain excitatory neurons did not affect body weight growth in female mice, although a reduction of NPY-1r mRNA expression was observed in CA1, CA3, DG of $NPY1r^{rfb}$ females as compared to controls. Furthermore, NPY-1r mRNA expression was significantly reduced also in the hypothalamic PVN and medial nucleus of the amygdala (MeA): such a reduction in NPY-1r expression in PVN and MeA was not observed in $NPY1r^{rfb}$ males (Paterlini et al., 2021). The differential pattern of NPY-1r inactivation in females and males might be responsible for the sex differences observed in the role of limbic NPY-1r in the regulation of body weight.

Based on present and previous studies, we confirm that limbic NPY-1r is differently regulated in males and females and its actions are strongly dependent upon sex. We also demonstrated that, especially in males, the different effects seem to be linked to the early environment and the amount of maternal care received during first postnatal days.

In the second experiment we exposed $NPY1r^{2lox}$ and $NPY1r^{rfb}$ females reared by Balb/c, C57Bl/6J, CD-1 and FVB/J foster mothers to a reproductive challenge. Females of both genotypes were mating with C57Bl/6J wild-type males and behavioral tests were performed during pregnancy. We observed that females seem to be resilient to metabolic and behavioral effects of the conditional deletion of NPY-1r gene. We hypothesized that genotype and the early maternal environment can affect reproduction or can exerts an effect on physiological state of pregnancy.

The most interest findings is that regardless to the strain of foster dams, $NPY1r^{rfb}$ females showed lower reproductive success compared to $NPY1r^{2lox}$ females, that means reduced pups’survival evaluated at PND 6. The reasons of the decreased survival rate of pups were not known. One of the possible explanations is an incorrect interaction between mother and pups, due to an alteration in maternal or nurturing behavior, or in social recognition of mother toward pups. The observation of pups scattered around the cage, without milk in their stomach, ignored by dams supporting this supposition. Deficit of social recognition of pups were observed also in mouse model of autism syndrome (Grabrucker et al., 2021). Another possible explanation could be a reduction in nurturing behavior by dams, that spent more time out of the nest compared to NPY^{2lox} females (Ladyman & Woodside, 2009).

We cannot exclude that higher mortality of offspring could be due to an olfactory alteration of mothers that induce the reduction of the ability of dams to recognize pups and to develop maternal and nurturing behavior (Ehret & Buckenmaier, 1994) or in shortage of milk production/ejection by the mother or due to inadequate suckling stimulus by pups (Wall & McFadden, 2012). Offspring from NPY1r^{rfb} seem to be less active and characterized by a lower body weight at birth compared to NPY1r^{2lox} pups, although no differences were recorded in litter size. Analysis of days from mating to delivery showed no differences due to genotype in Balb/c, C57Bl/6J and FVB/J mothers, while shorter time to give birth were observed in NPY1r^{rfb} females reared by CD-1.

The second aim of the experiment was to assess if the conditional deletion of limbic NPY-1r might affect behavior of females during pregnancy. Anxiety-like behavior were evaluated through the OPF test performed in two different times: before mating (baseline) and pregnancy. In general line, both the genotype showed high levels of anxiety-like behavior, with a higher amount of time spent in border zone compared to time spent in medium and center zone.

Results showed that regardless of genotype or adoption, during pregnancy females display a decrease in locomotor activity in order to reduce energy expenditure to facilitate a positive energy balance (S R Ladyman et al., 2020). Furthermore NPY1r^{rfb} females reared by CD-1 dams spent less time on center zone of the arena compared to NPY1r^{2lox} females. And pregnancy induce a reduction in time spent on the center zone compared to the baseline. Females of both genotypes reared by C57Bl/6J spent more time in the medium zone during pregnancy compared to the baseline. This data suggests that pregnancy has only partially effect on anxiety-like behavior of females.

When tested for depressive-like behavior NPY1r^{rfb} females reared by C57Bl/6J and FVB/J foster mothers showed a lower consumption of sucrose solution compared to NPY1r^{2lox} dams, while no differences due to genotype were recorded in females reared by Balb/c and CD-1 foster mothers. The observation of decreased sucrose solution consumption by NPY1r^{rfb} suggests that a depressive-like state could lead in behavior of knockout females toward pups. When we perform nest building assessment test, we observed no differences in percentage of dams with an high quality of built nest reared by HABN foster mothers, instead of females reared by LABN, in which an high percentage of NPY1r^{rfb} achieved a nest a nest score $\geq$4. Interestingly, when we performed retrieving test, we observed a significant difference in latency to retrieve the first pup in the nest regardless to strain of foster mothers, NPY1r^{2lox} showed lower retrieval latency time compared to NPY1r^{rfb} dams.

These results showed that conditional deletion of limbic NPY-1r during the reproductive challenge affects only partially the behavioral profile of females in anxiety-like and depressive-like behavior but exerts an effect in retrieving pups by mothers. Moreover, early maternal environment does not seem to be a strong influence on these effects. Conversely, reduced expression of NPY-1r effects were recorded in offspring features: $NPY1r^{rfb}$ females regardless to adoption strain showed lower body weight of pups at birth, and a decreased reproductive success compared to $NPY1r^{2lox}$ females.

In the third experiment we performed cross-fostering procedure between $NPY1r^{2lox}$ and $NPY1r^{rfb}$ females reared by CD-1 dams, in order to assess if cross-fostering could exert an effect on reproductive success. We perform the same sequence of behavioral test scheduled for experiment 2. At delivery, we created 4 experimental groups: $NPY1r^{2lox}$ and $NPY1r^{rfb}$ non cross-fostered (left undisturbed with their own pups) and $NPY1r^{2lox}$ and $NPY1r^{rfb}$ reciprocally cross-fostered. Interestingly, cross-fostering procedure induce an increase in reproductive success in $NPY1r^{rfb}$ females (18,75% vs 50% of dams with at least one pup at PND 6) and a decrease in reproductive success in $NPY1r^{2lox}$ females (80% vs. 50% of dams with at least one pup at PND 6).

This result suggest that survival rate of pups probably depends on a combination of factors that involved not only the mothers, but also pups. In conclusion, in this work, we demonstrated that the conditional deletion of NPY-1r induce different effects, in sex-dependent manner, contributing to develop a sexual dimorphic phenotype.

Reduced limbic expression of NPY-1r in males affect agonistic and anxiety-like behavior, besides metabolic functions, increasing the susceptibility to diet-induced obesity. In contrast, females were “shielded” by the actions of sexual hormones, in development of the effects observed in males. However, when challenged for reproduction, females showed a drastic decrease in reproductive success, and a lower body weight of pups at birth, suggesting that limbic NPY-1r expression affect physiological reproductive functions.

Nevertheless, further studies are needed to better understand the impact of conditional deletion of limbic NPY-1r and early maternal environment on reproduction and the mechanisms underlying the effects observed in females are carried out.

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