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**NEUROPEPTIDE Y SYSTEM IN MOOD DISORDERS:
ROLE OF THE DIFFERENT RECEPTOR SUBTYPES**

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RIASSUNTO

L'obiettivo principale di questa tesi di dottorato è rappresentato dalla ricerca di nuove evidenze in grado di supportare la conoscenza di un possibile coinvolgimento del Neuropeptide Y (NPY) e dei suoi principali recettori Y_1, Y_2 e Y_5 nei meccanismi che regolano i disturbi dell'umore, quali depressione, ansia ed i disturbi legati all'esposizione allo stress. Questo studio, proposto dal dipartimento di Biologia del Centro Ricerche GlaxoSmithKline di Verona, è stato condotto nell'ambito di varie collaborazioni con centri universitari, quali il Karolinska Institutet di Stoccolma (Svezia), l'Ecòle Polytechnique Fédérale de Lausanne (Svizzera) ed il German Primate Center di Göttingen (Germania).

Il possibile ruolo di NPY e dei suoi recettori nella regolazione dei meccanismi implicati nella fisiopatologia dei disturbi dell'umore è stato analizzato utilizzando tecniche sperimentali sia *in vitro* che *in vivo* applicate a diversi modelli animali di depressione, ad un modello di topi transgenici ed a tessuti cerebrali umani post-mortem ottenuti da pazienti affetti da disturbi psichiatrici. In particolare si è focalizzata l'attenzione sull'analisi dell'espressione dei trascritti di NPY e dei suoi tre recettori mediante la tecnica dell'ibridazione *in situ* applicata a tre modelli animali di depressione: i ratti Flinders Sensitive Line, un modello genetico particolarmente interessante data l'influenza della componente genetica in questo tipo di disturbi, il "chronic mild stress" ed il "chronic social defeat", due modelli di stress; quest'ultimo ritenuto una tra le maggiori cause dei disturbi di depressione ed ansia. Il chronic social defeat è stato studiato su due diverse specie animali: un roditore - il ratto - ed un non roditore - la tupaia o tree shrew (*Tupaia belangeri*) - considerata la sua elevata omologia genetica con l'uomo. I modelli utilizzati sono serviti ad approfondire lo studio del coinvolgimento del sistema di NPY nei disturbi dell'umore cercando di chiarire i meccanismi attraverso cui questo sistema neuropeptidergico agisce e provando a dimostrare quale dei principali sottotipi recettoriali abbia un ruolo di rilievo nella fisiopatologia di questi disturbi e nei meccanismi di regolazione delle risposte agli stress. Lo studio effettuato evidenzia che i tre sottotipi recettoriali sono differentemente espressi nei vari modelli animali e subiscono variazioni dell'espressione dei loro trascritti di tipo specie-specifico. Si è dimostrato che i tre recettori sono diversamente influenzati dall'esposizione ai vari tipi di stress, tuttavia, sebbene il sottotipo Y_5 sembri essere particolarmente affetto da variazioni trascrizionali nei modelli animali considerati, non è stato ancora completamente chiarito quale sia il sottotipo recettoriale maggiormente coinvolto nella regolazione dei disturbi dell'umore. Il coinvolgimento del recettore Y_2 in tali disturbi non sembra essere

sostenuto da questa ricerca, sebbene numerosi studi preclinici e analisi di tessuti umani post-mortem avessero dimostrato un ruolo di questo recettore nell'ansia e nella depressione. In generale, in questo studio le variazioni più consistenti e frequenti di espressione del trascritto di NPY e dei suoi recettori sono state osservate a livello dell'ippocampo, dell'ipotalamo e dell'amigdala, fornendo ulteriore supporto all'importanza cruciale di tali regioni cerebrali nella fisiopatologia dei disturbi affettivi.

Al fine di confermare alcuni precedenti studi comportamentali che avevano dimostrato che la delezione del recettore Y_2 era in grado di indurre una riduzione dello stato d'ansia ed un aumento della capacità di risposta agli stress, è stato condotto uno studio *in vivo* sottoponendo alcuni topi transgenici, ai quali era stata effettuata una delezione completa di tale recettore, a test di depressione ed ansia comunemente utilizzati. In contrasto con i risultati precedenti, lo stato d'ansia e lo stato depressivo di tali topi non hanno subito variazioni significative rispetto ai loro controlli sebbene i topi utilizzati in entrambi gli studi fossero stati completamente privati dello stesso recettore: sembra quindi non essere possibile supportare un ruolo diretto del recettore Y_2 nei meccanismi che regolano disturbi quali depressione ed ansia. Una possible causa del diverso comportamento legato agli stati d'ansia e di depressione è da ricercarsi nei diversi ceppi a cui i topi analizzati nei due studi appartenevano. L'analisi ha dimostrato l'importanza della scelta del ceppo degli animali: una diversa componente genetica tra i vari ceppi può avere un impatto maggiore sul fenotipo dell'animale rispetto alla delezione di un intero gene e questo fenomeno suggerisce quindi una certa cautela nella scelta degli animali e nell'interpretazione dei dati forniti da animali transgenici.

Infine, l'espressione del trascritto del recettore Y_2 è stata analizzata attraverso la tecnica dell'ibridazione *in situ* a livello dell'amigdala e della regione corticale ad essa adiacente in tessuti umani post-mortem ottenuti da pazienti affetti da diversi disturbi psichiatrici e paragonata all'espressione in soggetti di controllo, in cui tali disturbi non sono stati diagnosticati. Anche in questo caso non si è confermato il ruolo di Y_2 nei meccanismi che regolano la fisiopatologia dei disturbi affettivi, dato che non è stata dimostrata alcuna differenza tra i diversi gruppi patologici nell'espressione di tale recettore a livello dell'amigdala. Inoltre, l'espressione del recettore Y_2 è stata studiata in relazione al consumo di sostanze d'abuso. Differentemente dagli utilizzatori di marijuana, una variazione del trascritto di tale recettore è stata osservata nei soggetti consumatori di cocaina e nicotina, ipotizzando un coinvolgimento di Y_2 nei meccanismi che regolano l'assunzione di queste sostanze. Riguardo all'etanolo, questo studio non ha dimostrato alcuna variazione

trascrizionale del recettore Y_2 in soggetti che ne facevano uso, non potendo confermare alcuni studi che sostengono l'importanza del ruolo del sistema di NPY nella dipendenza da etanolo.

La presente tesi ha fornito nuovi interessanti dati riguardanti il coinvolgimento del sistema del Neuropeptide Y nei disturbi affettivi, tuttavia per completare lo studio sarebbero necessari ulteriori approfondimenti in particolare sui tessuti umani. Disturbi quali la depressione rappresentano infatti una condizione tipicamente umana, non esattamente riproducibile nei modelli animali, che possono solamente fornire una semplificazione dello stato depressivo. L'utilizzo di composti antagonisti recettoriali selettivi applicato a studi su modelli animali sarebbe in grado di fornire risultati più dettagliati e specifici che potrebbero meglio indicare il sottotipo recettoriale maggiormente coinvolto in questi disturbi, fornendo così un nuovo bersaglio per il possibile sviluppo di nuovi farmaci ansiolitici ed antidepressivi. Ad oggi questa indagine sembra essere piuttosto difficoltosa da effettuare data la mancanza di composti selettivi verso un sottotipo recettoriale e con caratteristiche tali da essere capaci di agire a livello del sistema nervoso centrale. La sintesi di questo genere di composti permetterebbe di comprendere maggiormente la base dei meccanismi che regolano il funzionamento del sistema di NPY nei disturbi dell'umore e di fornire un trattamento efficace contro l'ansia e la depressione.

ABBREVIATIONS

5-HT	5-hydroxytryptamine
AB	accessory basal nucleus
ACTH	adrenocorticotropin hormone
ANCOVA	analysis of covariance
ANOVA	analysis of variance
ARC	arcuate hypothalamic nucleus
BI	basal intermediate division
BMC	basal magnocellular division
bp	base pairs
CA1	hippocampal CA1 region
CA2	hippocampal CA2 region
CA3	hippocampal CA3 region
CC	cingulate cortex
CCK	cholecystokinin
cDNA	complementary DNA
CMS	chronic mild stress
CNS	central nervous system
CRF	Corticotrophin-releasing factor
CSF	cerebrospinal fluid
Ct	threshold cycle
CUS	chronic unpredictable stress
DG	dentate gyrus
DSM	Diagnostic and Statistical Manual of Mental Disorders
EC	entorhinal cortex
ECS	electroconvulsive stimuli
ECT	electroconvulsive treatment
EPM	elevated plus maze
FRL	Flinders Resistant line
FSL	Flinders Sensitive line
FST	forced swim test
GABA	γ-ammino butirric acid
GAD	generalized anxiety disorder
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
HA	high anxiety
HPA	hypothalamic-pituitary-adrenal
i.c.v.	intracerebroventricular
i.p.	intraperitoneally
IRMA	immunoradiometric assay
KO	knockout
L	lateral nucleus
LA	low anxiety
LABORAS	Laboratory Animal Behaviour Observation Registration and Analysis System
LHPA	limbic-hypothalamic-pituitary-adrenal
MDD	major depressive disorder
MeA	medial amygdala
mRNA	messenger RNA
NA	Noradrenaline
NK	Neurokinin
NPY	Neuropeptide Y
NPY-LI	NPY-like immunoreactivity
N.S.B.	non specific binding
PBS	phosphate buffered saline
PMI	post-mortem interval
PP	pancreatic polypeptide
PYY	peptide YY
RIA	radioimmuno assay
RT	reverse transcription
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RT-qPCR	Real Time-quantitative Polymerase Chain Reaction
S	septum
SNRI	serotonin-noradrenergic re-uptake inhibitor
SP	Substance P
SRI	selective re-uptake inhibitor
SSC	saline sodium citrate
SSRI	selective serotonin re-uptake inhibitor
T.B.	total binding
TC_d	deep layers of the temporal cortex
TC_{sup}	superficial layers of the temporal cortex
TH	thalamus
VMH	ventral medial hypothalamic nucleus
VMHDM	ventro-medial hypothalamus dorso-medial portion
WT	wild type

1. INTRODUCTION

1.1 DEPRESSION

Depression has been described by mankind for several millennia. The term melancholia, which means “black bile” in Greek, was first used by Hippocrates around 400 b.C. (Akiskal *et al.,* 2000). The major symptoms of depression and the comorbidity of depression with anxiety and excessive alcohol consumption were recognized in ancient times, indeed similarities between ancient descriptions of depression and those of the modern *era* are striking. From the middle part of the 19th century the brain became the focus of the efforts to understand the pathophysiology of depression.

Today, depressive disorders represent a common psychiatric disorder experienced by more than 10% of the population at least once during the lifetime (Blazer *et al.,* 1994). Women are more prone to the disease than men, with almost a two-fold lifetime prevalence rate: around 21% of women and 13% of men of the Unites States population. The mortality is high: 70% of all suicides can be attributed to depressive disorders and they represent a major cause of morbidity worldwide: studies in the United States suggest that 2–3% of the population is hospitalized or seriously impaired by affective illnesses (Blazer, 2000). The World Health Organization has declared depression as the single largest cause of morbidity for women and the leading cause of disability worldwide. Despite the devastating impact of depressive disorders, little is known about their etiology and pathophysiology.

1.1.1 Major Depressive Disorder

Since the 1960s, depression has been diagnosed as Major Depressive Disorder (MDD), as defined by the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV, 2000). The diagnosis of depression is not based on objective diagnostic tests, but rather on a highly variable set of symptoms, described by the DSM-IV as: emotional dysregulations, such as sadness, emotional instability, irritability, anhedonia and anxiety; cognitive symptoms, such as impaired concentration, attention and short-term memory deficits, and a pervasive negative distortion, leading to a pessimistic cognitive interpretation of everyday experiences. The combination of emotional and cognitive symptoms often leads to a passive or active desire to die. These symptoms are frequently accompanied by severe endocrine symptoms, such as change in sleep, appetite and sexual behaviour, as well as behavioural manifestations, such as motor retardation and inertia. MDD is also associated with metabolic

changes leading to an increased risk for a number of somatic problems, such as osteoporosis and cardiovascular disorders; in fact MDD leads to a 4-5-fold increase in the risk for myocardial infarction (Pratt *et al.,* 1996), resulting in a markedly increased morbidity and mortality in individuals with MDD.

Patients affected by MDD have a mean of 4-5 episodes during life, each of which has a mean duration of 5-6 months (Eaton *et al.,* 1997). Only 10 to 20% of patients have no more than a single episode. The etiology of MDD is particularly complicated: various factors, such as acute and chronic stress, genetic factors, early life trauma and somatic diseases have been associated with the disorder.

1. *Acute stress*. One of the factors eliciting MDD is acute stress (adverse life experiences). The majority of first episodes of MDD are preceded by an acute stress experience or adverse life event (Mazure *et al.,* 2000). However, the importance of life events in eliciting a major depressive episode diminishes as the number of depressive episodes increases in a given subject (Kendler *et al.,* 2000).

2. *Chronic stress*. Chronic stress or problematic life circumstances is a second known cause of MDD. Chronic stress associated with a psychological dimension of ‘‘entrapment’’ (being trapped between apparently unsolvable psychosocial difficulties or demands) or a dimension of ‘‘humiliation’’ is reported to be particularly depressogenic (Kendler *et al.,* 2003). Whether acute or chronic stress leads to MDD is also dependent on the individual. The liability to develop depression in response to stress is highly variable between individuals; in fact, genetic factors and early traumatic experiences are considered very important etiological factors.

3. *Genetic factors*. Family studies report that the risk for MDD is increased in first-degree relatives of MDD patients. Earlier onset seems to be associated with increased familiarity and the heritability estimate range goes from 37 to 70%. Twin studies consistently show a genetic component in the vulnerability for the disease: the probability that a twin will develop depression, given that the other twin has depression, is about 14-24% for dizygotic twins and about 54-65% for monozygotic twins (McGuffin & Katz, 1989). These evidences indicate interplay between genetic and environmental factors in the etiology of MDD. The exact nature of this interplay has not been elucidated, but most probably genetic factors influence the risk to develop MDD partly by altering the sensitivity of individuals to the depression-inducing effect of stressful life events. The best-fitting model for the joint effect of stressful events and genetic liability on the onset of MDD has suggested genetic control of sensitivity to the depression-inducing effects of stressful life events.

4. *Early life trauma*. Severe adverse experiences in early life, such as child abuse, increase the vulnerability for MDD and for a number of anxiety disorders later in life (Duncan *et al.,* 1996). 20% of women who had been exposed to sexual abuse as a child had psychiatric disorders, predominantly depressive in type, compared with 6.3% of the non-abused population (Mullen *et al.,* 1988). A history of physical or sexual abuse in childhood is often associated with MDD, with reversed neurovegetative features (Levitan *et al.,* 1998).

5. *Somatic diseases*. Somatic diseases can be associated with a strongly increased prevalence of MDD. The disease can act as a non specific stressor; however, in a number of cases, MDD often occurs before the onset of the somatic disease, indicating a more direct biological link between them. Ischemic heart disease, renal failure, infectious diseases and organic brain disorders show a high co-morbidity with MDD. In most cases, untreated MDD worsens the prognosis of the somatic disorder (Claes, 2004).

1.1.2 Brain structures related to depression

Many brain areas implicated in the regulation of emotions mediate the diverse symptoms of depression. The neural circuitry associated with emotional processing is complex and overlaps significantly with the limbic system. The anhedonia and the reduced motivational that predominate in many depressed patients (Drevets, 2001) could be mediated by the so called reward pathways in the brain, including regions like medial prefrontal cortex, striatum (in particular the ventral part or nucleus accumbens) and ventral tegmental area.

Among the brain regions known for their prominent function in the pathophysiology of depression and anxiety, the amygdala, a structure consisting of several nuclei, is part of the limbic system and plays a critical role for the regulation of affective behaviour and neurochemical responses to stress, suggesting a strong involvement of this limbic area in anxiety, fear and emotional memory (Aggleton, 1993; Gallagher & Chiba, 1996; LeDoux, 2000). In various animal models, electrical stimulation of the amygdala produces behavioural changes similar to those produced by stressful or fearful stimuli and lesions of the amygdala block innate or conditioned reactions to stress (Aggleton, 1993; Davis *et al.,* 1994).

In humans, functional studies on normal individuals and on brain-damaged patients indicate a key role of the amygdala on the processing of emotionally and socially relevant information in response to aversive visual stimuli (Adolphs *et al.,* 1998; Morris *et al.,* 1998). Functional and morphological alterations of the amygdala have also been described with imaging techniques in depressed patients (Chen *et al.,* 2007; Fales *et al.,* 2007; Drevets *et al.,* 1992; Drevets & Raichle, 1992; Drevets, 2001, 1999; Frodl *et al.,* 2002). Increased cerebral

blood flow and glucose metabolism have been reported in different forms of depression (Abercrombie *et al.,* 1998; Drevets *et al.,* 1992), as well as increased amygdala volume (Frodl *et al.,* 2002), although some contrasting results have also been described (Sheline *et al.,* 1998). A brain region reciprocally connected to the amygdala is the prefrontal cortex, which plays a relevant role in the neuropathology of psychiatric disorders, regulating the social behaviour and cognitive functions, allowing cognitive control of conditioned fear (Drevets, 2001, 1999). These two regions share afferent and efferent connections with the hippocampus, another limbic region, which plays a well documented role in declarative memory and spatial learning (Barco *et al.,* 2006; Manns & Eichenbaum, 2006). Deficits in the hippocampus-dependent cognitive functions are evident in depressed patients, supporting a role for dysfunctions of this brain region in depression (Bremner *et al.,* 2000; Bremner *et al.,* 2004; Vythilingam *et al.,* 2004; Gould *et al.,* 2007). Prefrontal cortex and hippocampus mediate the cognitive aspects of depression, such as memory impairments and feeling of worthlessness, hopelessness, guilt and suicidality (Nestler *et al.,* 2002). These various brain areas operate in a series of highly interacting parallel circuits, which could represent a neural circuitry involved in depression.

Finally, the neurovegetative symptoms of depression, including alterations of sleep, appetite, energy, loss of interest in sexual and other pleasure activities have been associated to different hypothalamic nuclei (Nestler *et al.,* 2002).

1.1.3 Pharmacological treatment of depression

In contrast to the limited understanding of the pathophysiology of depression, there are many antidepressive treatments available. Around 70% of people with depression respond to currently available antidepressant medications or electroconvulsive treatment (ECT), a procedure used to treat depression in pharmacotherapy-resistant cases or in other situations, such as early pregnancy, elderly or severe hepatic or renal diseases, in which drug metabolism and excretion are impaired. In addition, several forms of psychotherapy can be effective for mild to moderate cases and the combination of medication and psychotherapy can exert a synergistic effect. However, antidepressant medications have a therapeutic effect delayed of several weeks or even months, whereas the side effects are manifested within hours or days following the beginning of the administration.

1.1.3.1 Overview of current antidepressant treatments

The use of antidepressant drugs in the treatment of depression dates back to the early 1950s,

with the discovery by serendipity of two classes of effective antidepressants: the tricyclic antidepressants and the monoamine oxidase inhibitors. Both classes of antidepressants have been demonstrated to effectively improve the symptoms of depression. Thus, for the first time, depression was treated pharmacologically; whereas earlier it had been only treated with ECT. Both classes of antidepressants have been found to increase the brain extracellular concentrations of the two neurotransmitters serotonin or 5-hydroxytryptamine (5-HT) and noradrenaline (NA), implicated in the mechanisms involved in the pathophysiology of depression by blocking their re-uptake back to nerve endings or by inhibiting the main metabolizing enzyme, monoamine transporter or monoamine oxidase, respectively. As drugs that alleviate depression by increasing extracellular monoamine levels, it has been proposed that depression might be produced by a 5-HT or NA deficiency in functionally important receptor sites in the brain: this proposal constitued the basis for the "monoamine hypothesis of depression".

Successively, in the early 1980s, a second-generation of drugs, the selective reuptake inhibitors (SRIs), was developed to enhance the function of specific monoamine systems by increasing the availability of monoamines through blockade of the presynaptic transporters that promote the re-uptake, thus increasing the transmitter availability in the synaptic cleft. The improved tolerability of these new drugs led to the development of fluoxetine, a selective serotonin reuptake inhibitor (SSRI); more recent SRIs are serotonin noradrenergic re-uptake inhibitors (SNRIs). These antidepressant drugs target the monoamine system and are similar in terms of efficacy and delay in the onset of antidepressant effect. In fact, there is a time lag of weeks to months in the desired therapeutic response, while the molecular inhibition of 5-HT or NA re-uptake occurs immediately. This therapeutic lag means that enhance serotonergic or noradrenergic neurotransmission *per se* is not responsible for the clinical actions of these drugs. Finally, despite the advances in the psychopharmacology, the treatment of depressive disorders is still not satisfactory, because all the currently used pharmacological substances are affecting the monoamines in the central nervous system (CNS).

Recent advances in the understanding of the molecular and cellular functions of neurons have led to the identification of additional extracellular and intracellular signaling targets (Nestler *et al.,* 2002). Such potential targets include neurotransmitter receptors, neuropeptides, intracellular second messenger generating systems and proteins involved in the neurotrophic cascades (neurogenesis and apoptosis), glutamate modulating agents, substances affecting melatonin and others modulating the hypothalamic-pituitary-adrenal

(HPA) axis (Pacher *et al.,* 2001). New classes of antidepressants acting on these pathways represent the new-generation of antidepressants with novel mechanism of action.

1.2 ANIMAL MODELS OF DEPRESSION

The animal models of depression represent a valid research tool to explore the possibility that a neurotransmitter system has a role in depression. The reason for using animal models of depression is based mainly on the fact that antidepressants are largely devoid of mood-elevating effects in normal individuals, meaning that the relevance of the studies carried out in normal animals is questionable and thus animal models of depression are indispensable in research. Therefore, the effects of antidepressant treatments on neurotransmitters in healthy rats are not necessarily informative of their therapeutic mechanism of action and do not necessarily contribute to understanding the pathophysiology of the affective disorders. However, there are only a few studies in which "depressed" animals have been used (Kornstein, 1997; Frackiewicz *et al.,* 2000).

A valid animal model of depressive disorders should reflect the etiology and replicate symptoms, course and treatment of human depression. To assess the validity of an animal model, three general headings are evaluated: (1) *face validity*, the apparent similarity between the behaviour observed in the model and the specific symptoms of depression; (2) *predictive validity*, concerning how the model respond to drugs that are clinically active and (3) *construct validity*, implying that human and animal responses are homologous and thus the response observed has clinical significance for the disorder modeled. The rationale of these three sets of criteria of validation of the animal models of depression has been discussed in detail (McKinney & Bunney, 1969; Willner 1991, 1986, 1984).

Different animal models of depression have been developed, such as stress models (e.g. learned helplessness, chronic unpredictable stress and behavioural despair), genetic models (e.g. the Flinders Sensitive Line (FSL) and the Fawn-Hooded rats), lesion models (e.g. olfactory bulbectomy), separation models (e.g. maternal separation) and developmental models (e.g. neonatal antidepressant treatment, prenatal/neonatal stress).
In the present study, a genetic model and two stress models were considered.

1.2.1 Genetic models

The selective breeding is an approach used to generate genetic models of depression, such as the FSL rats, based on the assumption that depression in humans generally requires a genetic- and/or environmental-based vulnerability.

1.2.1.1 Flinders Sensitive Line rats

Numerous evidences have suggested that depressive disorders are caused by genetically defined neurochemical alterations in the CNS and an example of a putative genetic animal model of depression is represented by the FSL rats. These rats and their control Flinders Resistant Line rats (FRL) are Sprague-Dawley derived and selected for the high – FSL – or low – FRL – sensitivity to the anticholinesterase agent diisopropyl fluorophosphate (Overstreet *et al.,* 1979). The FSL rat supersensitivity to cholinergic agents is consistent with the cholinergic hypothesis of depression (Janowsky *et al.,* 1972). The FSL rats display features similar to those observed in depressed humans: reduced body weight, elevated REM sleep and reduction in REM sleep onset (Shiromani *et al.,* 1988), reduced basal motor activity (Overstreet & Russell, 1982), increased immobility and anhedonia after exposure to stressors (Pucilowski *et al.,* 1993), disturbance in learning, submissiveness and decreased response to rewards (Overstreet, 1993). However, there is no evidence for anhedonia and cognitive disturbance, core observations in depressive disorders, but there are some evidences that the resemblance of the FSL rats to depressed individuals may be higher if environmental manipulations are induced (Overstreet *et al.,* 2005). Under basal conditions, no differences in anhedonia are detected, but the FSL rats exhibit a greater decrease in saccharin intake following the application of chronic mild stress (Pucilowski *et al.,* 1993). These data imply that the FSL rats may be considered a model of predisposition to depression, rather than a model of depression *per se*. Moreover, they respond to different chronic, but not acute, antidepressant treatments, such as tricyclic and SSRIs, with a reduction of immobility in the forced swim test, a predictive test for antidepressant activity (Porsolt *et al*., 1977). These rats, however, do not respond to bright light and lithium treatment (Overstreet, 1993; Overstreet *et al.,* 2005). The construct validity of the model derives from the cholinergic and serotonergic supersensitivity of the FSL rats, partially consistent with the cholinergic and serotonergic hypothesis of depression. Overall, the FSL rats meet reasonably well the criteria of face, construct and predictive validity for an animal model of depression, thereby making it a useful model to study the pathophysiology and pharmacology of depression (Yadid *et al.,* 2000).

1.2.2 Stress induced models

Stressful life events often contribute to the etiology of depressive episodes, thus stress induced animal models have been developed to study central nervous mechanisms leading to

depressive symptoms. The impact of stressful events on the development of psychopathologies has been largely investigated in pre-clinical studies and it has been evidenced that using stress to induce a feeling of loss of control might result in a behavioural state analogous to depression. Chronic stress models seem to have a major ethological value compared to the other models.

1.2.2.1 Chronic mild/unpredictable stress

This animal model was initially developed and set out because it can be considered valid as a simulation of depression and long-lasting in its duration. It was targeted at modelling anhedonia, the core symptom of the melancholic subtype of MDD in the DSM-IV, defined as the "decreased capacity to experience pleasure of any sort" (Fawcett *et al.,* 1983), and modelled by inducing a decrease in the responsiveness to rewards.

The chronic mild stress (CMS) protocol consists on the exposure of the rats to a variety of mild stressors (e.g. overnight illumination, periods of food and/or water deprivation, isolation housing, change of cage mate, cage tilt) which change every few hours over a period of weeks or months, resulting "unpredictable": thus this model is also called chronic unpredictable stress (CUS). The effectiveness of this procedure is usually monitored by tracking, over repeated tests, a decrease in the consumption of and/or preference for a palatable weak (1-2%) sucrose solution, a valid method to measure the sensitivity to rewards, thus demonstrating that a prolonged exposition to repeated stressors induces anhedonia (Willner, 2005, 1997a). In this way, the criterium of construct validity is fulfilled. Decreases in sucrose drinking cannot be related to non-specific changes in fluid consumption, since the intake of plain water is unaffected by CMS. Food intake is also not decreased, but CMS decreases food reward. In addition, CMS causes the appearance of many other symptoms of MDD, such as decrease in sexual, aggressive and investigative behaviours (D'Aquila *et al.* 1994) and reduced locomotor activity during the dark phase of the light-dark cycle (Gorka *et al.,* 1996). Furthermore, animals exposed to CMS show a variety of sleep disorders, including decreased REM sleep latency and increased number of REM sleep episodes and more fragmented sleep patterns (Moreau *et al.,* 1995). They also display a loss of body weight and show signs of increased activity in the HPA axis, including adrenal hypertrophy (Muscat & Willner, 1992) and corticosterone hypersecretion (Ayensu *et al.,* 1995) and finally abnormalities in the immune system. All these observed behavioural changes may be maintained for several months and contribute to fulfil the face validity criterium. However, normal behaviour is restored, during continued application of CMS, by chronic treatment

with a variety of antidepressant drugs, such as tricyclic or atypical antidepressants (Papp *et al.,* 1996). They are able to reverse anhedonia and increase responsiveness to rewards in 3-4 weeks of treatment, which closely resemble the clinical time course of antidepressant action, making possible the validation of the CMS model, because the criterium of predictive validity is also fulfilled (Willner *et al.,* 1987).

1.2.2.2 Chronic social defeat stress in rat and tree shrew

The most common stressors in human subjects are of a psychological or social nature. The loss of position within the social organization is characterized in human population by feelings of worthlessness and inadequacy, assumed to arise from negative self-evaluation (Bjorkqvist, 2001; Kessler, 1997; Kessler *et al.,* 1985b). Many animal species develop hierarchical social structures based on agonistic encounters between males and therefore the loss of social position provides a submissive behaviour. A number of studies have showed that subordination stress (social defeat) is an important factor that may lead to psychopathological changes (Bjorkqvist, 2001; Fuchs & Flugge, 2002).

Social defeat in rats or in a non-rodent species, the tree shrews (*Tupaia belangeri)*, using the resident-intruder paradigm (Tornatzky & Miczek, 1994), represents a suitable and naturalistic experimental paradigm to study the causal mechanisms of major depression (Fuchs *et al.,* 1996). In this paradigm, an adult male - the intruder - is introduced into the home cage of an unfamiliar, aggressive individual - the resident -. The animals interact rapidly, fight and the intruder usually loses the encounter; as a consequence, a social hierarchy is established with a dominant and a subordinate male. The animals are then physically separated, but they constantly remain in olfactory, visual and acoustic contact for the rest of the stress session. The direct physical contact is only allowed for approximately one hour every day, while in the remaining time the two animals are separated by a wire mesh barrier that does not interfere with the hierarchy. These conflict conditions can last several days or even weeks and they are perceived as highly stressful by the subordinated animal. Subordinated animals exhibit physiological and behavioural modifications correlated to the depression state, including decreased locomotor and exploratory activity (Koolhaas *et al.,* 1997b; Meerlo *et al.,* 1996), reduced self-grooming (van Erp *et al.,* 1994), impaired consumatory behaviour and consequently loss of body weight (Rybkin *et al.,* 1997; Kramer *et al.,* 1999), reduced aggression and sexual behaviour (McGrady, 1984), increased submissive behaviour and anxiety (Ruis *et al.,* 1999). Moreover, social defeat alters the sensitivity to subsequent challenges of other kinds of stress, impairs anticipatory behaviour

(Von Frijtag *et al.,* 2000) and induces cross-sensitization to psychostimulants (Kapur and Mann, 1992). Physiologically, the defeated animals show increased adrenocorticotropin hormone (ACTH) and glucocorticoid activity (Buwalda *et al.,* 2001, 1999), altered circadian rhythms in heart rate, blood pressure and core temperature (Meerlo *et al.,* 1996; Sgoifo *et al.,* 1999), disturbances in sleep (Rüther, 1989), impaired immunological function and reduced resistance to diseases (Engler *et al.,* 2004; Stefanski & Engler, 1998). In addition, social defeat produces a variety of changes in neurotransmitter systems, including altered dopamine turnover in different brain areas (Isovich *et al.,* 2001), changes in γ-ammino butirric acid (GABA) (Miller *et al.,* 1987), glutamate (Krugers *et al.,* 1993) and serotonin receptor binding (McKittrick *et al.,* 2000) and affect opioid system (Miczek, 1991; Coventry *et al.,* 1997).

A great number of these findings derive from studies performed on tree shrews (*Tupaia belangeri*), phylogenetically regarded as an intermediate between insectivores and primates (Martin, 1990). They present a high genetic homology with humans (90-98% amino acid sequence identity, whereas the homology with the rat is only about 80%). They are solitary and the male show a pronounced territoriality, used to establish naturally occurring challenging situations under experimental control. Moreover, tree shrews are day-active, so their biological rhythms might be more similar to those of humans than the diurnal rhythms of night-active rodents such as rats.

The observed behavioural and neuroendocrine changes in socially stressed animals mimic depressive-like symptoms, comparable to those of depressed patients. Thus, the chronic social stress model has obvious face and construct validity for depression. Interestingly, it has been demonstrated that antidepressants, such as the tricyclic clomipramine, lead to a time-dependent improvement of these symptoms, restoring the endocrine and behavioural parameters (Fuchs et al., 1996; Kramer et al., 1999). Thus, the predictive validity is also fulfilled, making the chronic social stress a validated animal model of depression.

1.3 ANXIETY

The DSM-IV describes the anxiety neurosis as divided into multiple syndromes, such as generalized anxiety disorder (GAD), the most provisional anxiety syndrome, obsessive-compulsive disorder, post traumatic stress disorder, social anxiety disorder and panic disorder.

Anxiety disorders in general are the most common form of mental illness in the United States (DuPont *et al.,* 1996): several community-based surveys have estimated the current prevalence rates for GAD at 1.2% to 6.4%, with a lifetime prevalence of 5.1% in the adult population (Kessler *et al.,* 1994). Epidemiology and clinical studies have suggested that GAD typically occurs before the age of 40, runs a chronic, fluctuating course and affects women twice as often as men (Walley *et al.,* 1994).

Anxiety symptoms include motoric, autonomic and cognitive manifestations, with an excessive, unrealistic and uncontrollable worry about a number of events or activities as the key feature, occurring for at least six months; a core set of increased arousal and motor tension symptoms have been also identified (Brown *et al.,* 1994). The anxiety and worry need to be associated with an unspecified number of the following symptoms: motor tension (restless, tension headaches, trembling, inability to relax), being easily fatigued, irritability, apprehension (worry about future, feeling “on edge”, difficulty concentrating), autonomic over-activity (tachycardia, epigastric discomfort, dizziness, dry mouth, sleep disturbance). The anxiety, worry or physical symptoms cause clinically significant distress or impairment in social, occupational or other important areas of functioning.

Research studies have revealed the existence of a high rate of comorbid psychiatric disorders with GAD. In some studies, more than 90% of GAD patients fulfilled criteria for at least one or more concurrent disorders: strong association between GAD and other mood disorders, such as social phobia, major depression or dysthymia has been found. The presence of comorbidity has been also associated with hypomania and a high suicide attempt risk (Sanderson & Barlow, 1990). Studies examining order of onset of GAD and other comorbid disorders have suggested that onset of major depression seemed to follow the onset of anxiety (Fava *et al.,* 1992; Kessler, 2000). The presence of more than one psychiatric disorder may influence the diagnosis process and treatment response.

Studies have indicated that the vulnerability to develop GAD may be, at least in part, genetic. Twin studies have suggested a higher rate of concordance in monozygotic twins than dizygotic (Skre *et al.,* 1993). Moreover, other studies have demonstrated that while genetic

factors may predispose a person to GAD, environmental factors play an important role in the development of GAD (Kendler *et al.,* 1995 a,b).

1.3.1 Pharmacological treatment of anxiety

The first drugs used in the treatment of GAD were the benzodiazepines, generally used to treat neuroses rather than specific anxiety disorders (Davidson, 2001). They are active on the central GABA receptors, gated chloride channels, on which they act promoting the binding with the neurotransmitter, responsible of the opening of the receptor channels, inducing a cellular increase of chloride and an hyperpolarization of the cell membrane, inhibiting the release of other neurotransmitters (Shader & Greenblatt, 1993).

Abnormalities in noradrenergic system have also been implicated in the pathophysiology of anxiety disorders (Munjack *et al.,* 1990). Moreover, the serotonin system has been shown to play a role in fear and anxiety responses in animal models (Taylor *et al.,* 1985) and in humans (Kahn *et al.,* 1991; Garvey *et al.,* 1993). Thus, antidepressants have been consistently found to be equal or even superior in efficacy to benzodiazepines (Davidson, 2001). Studies are concordant in indicating that benzodiazepines are less effective than antidepressants in reducing psychic symptoms of GAD, while they may be more effective than these drugs in treating somatic symptoms (Rickels, 1982; Rickels & Schweizer, 1993). Even in patients without comorbid major depression, the presence of mild depressive symptoms may negatively affect the response of anxiety symptoms to benzodiazepines, but not to antidepressants (Rickels and Schweizer, 1993). For this reason, antidepressants should be favored over benzodiazepines as a first-line treatment for GAD (Ballenger, 2001) because of their efficacy against comorbid psychiatric disorders, such as depression. Moreover, the recurrence of anxiety symptoms in GAD patients occurs significantly more often with benzodiazepines in comparison with non-benzodiazepine anxiolytics (Ballanger, 2001).

Other examples of drugs used to treat anxiety disorders are represented by SSRIs and trcyclic antidepressants, more effective in improving the psychological symptoms, whereas the benzodiazepines lead to greater improvement in physical symptoms; moreover, β-blockers, beneficial in decreasing the autonomic symptoms of anxiety, especially cardiovascular manifestations, peptides and antipsychotic drugs are also included.

1.4 NEUROPEPTIDES

Recent research studies have focused on the neuroactive peptides as possible novel targets to study the mechanisms underlying the pathophysiology of depression and anxiety, in which neuropeptides could play a role, in view of their manifold interactions with monoamines, and the mechanism of action of the antidepressant drugs (Warnock *et al.,* 1998; Ressler & Nemeroff, 2000; Abelson *et al.,* 2007); thus, they have been considered an interesting source of new potential antidepressant molecules.

Neuropeptides have been grouped into families depending on common precursor, sequence homologies and often function. They have been demonstrated to be involved in a series of physiological functions and numerous studies have suggested a role for neuropeptides in the pathophysiology of a variety of psychiatric disorders. Regarding mood disorders, the Substance P (SP) or Neurokinin (NK), initially characterized for its role in pain, has been then demonstrated to have a role in depression and anxiety (McLean, 2005): interestingly, the efficacy of an antagonist of the SP preferential receptor, Neurokinin-1 (NK1), in the treatment of depression has been also demonstrated in a clinical trial (Kramer *et al.,* 1998). Cholecystokinin (CCK) is another neuropeptide abundantly expressed in the brain, which has been demonstrated to induce anxiety-related behaviour in animals (Harro *et al.,* 1993; Lydiard, 1994) and in humans (Bradwejn, 1992; Adams *et al.,* 1995; Kennedy *et al.,* 1999), acting predominantly through CCK-B receptors, mostly expressed in the CNS than the CCK-A subtype (Wank, 1995). Another important example is represented by the corticotropin-releasing factor (CRF), known for its involvement in the behavioural responses to stress (Koob, 1999; Koob & Heinrichs, 1999) and for its role in eliciting anxiety and fear responses when administered in various parts of animal brains (Butler *et al.,* 1990; Griebel, 1999). There are also evidences on the prominent role of Neuropeptide Y (NPY) in anxiety and depression, demonstrating that it induces anxiolytic-like effects when centrally injected (Heilig *et al.,* 1993; Heilig & Murison, 1987) and a depressive-like phenotype when it decreases both in human and animals (Widerlov *et al.,* 1988b; Widdowson *et al.,* 1992). The present study will provide further evidences for the involvement of the NPY system in response to stressful stimuli and for its role in the mechanisms regulating the pathophysiology of depression and anxiety.

1.5 NEUROPEPTIDE Y SYSTEM

1.5.1 Neuropeptide Y

1.5.1.1 Isolation and discovery

NPY was isolated from porcine brain extracts (Tatemoto *et al.,* 1982) and belongs to a family of related peptides, including pancreatic polypeptide (PP), peptide YY (PYY) and seminalplasmin (Herzog *et al.,* 1995). The members of this peptide family share some general characteristic, such as a chain length of 36 amino acids, a C-terminal amidation and α-amide structure. The unusual large number of tyrosine residues gave their names to NPY and PYY. Subsequent work have showed NPY to account for almost all the PP-like immunoreactivity in the brain (Lundberg *et al.,* 1984), while PYY has been mainly found in the intestine. Indeed, NPY is one of the most abundant peptides in the CNS and one of the most conserved peptides in evolution: the rat and human NPY sequences have been demonstrated to be identical (Larhammar *et al.,* 1987).

1.5.1.2 NPY distribution in the CNS

NPY is abundantly expressed in the rat and human brain (Adrian *et al.,* 1983; Chan-Palay *et al.,* 1986, 1985; Chronwall *et al.,* 1985). NPY has been found in two basic types of neurons: (1) short-axon cells or interneurons, thought to be elements of local inhibitory circuits and mainly found in the forebrain and (2) long projection neurons, mainly found in the brainstem, from which they project for a considerable distance (Hendry, 1993). Immunohistochemical studies have demonstrated a high degree of similarity between the distribution of NPY-like immunoreactivity (NPY-LI) in the rat and human brain (Chan-Palay & Yasargil, 1986; de Quidt & Emson, 1986 a, b). The highest concentrations of NPY-LI have been observed in periacqueductal gray, nucleus accumbens, hypothalamus, septum and amygdala, whereas lower amounts have been observed in the basal ganglia, globus pallidus, hippocampus and cortex. NPY is almost absent in pons and cerebellum. *In situ* hybridization studies have revealed the existence of a good correlation between the distribution of the NPY mRNA expression and the NPY-LI (Terenghi *et al.,* 1987; Chan-Palay *et al.,* 1988); (Fig. 1).

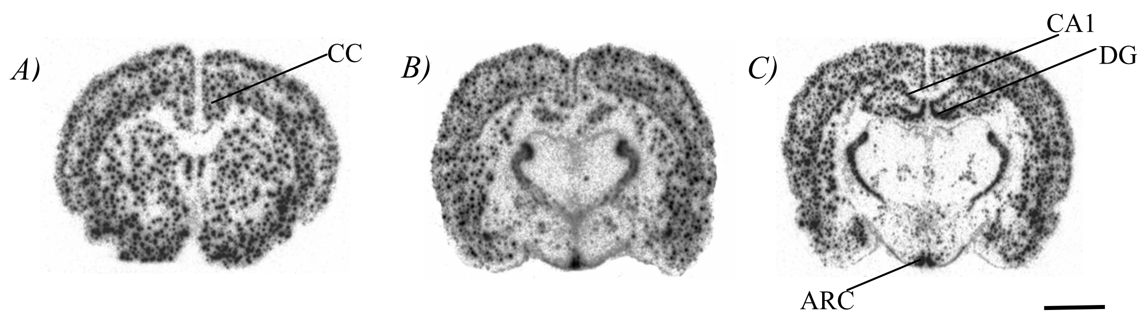


Fig. 1. *Representative images of the distribution of NPY mRNA expression in coronal sections of the rat brain, approximately at 1.60 mm (A), -1.88 mm (B) and -3.14 mm (C) from Bregma. Scale bar = 3.5 mm. CC, cingulate cortex; DG, dentate gyrus of hippocampus; CA1, hippocampal CA1 region; ARC, arcuate hypothalamic nucleus.*

1.5.1.3 Central functions of NPY

The wide distribution and abundance of NPY in numerous brain regions have suggested a variety of functions of this neuropeptide in the CNS. Several studies have investigated its role in the regulation of many central functions, revealing its involvement in various fundamental physiological processes. These include effects on feeding behaviour, water consumption, locomotion, body temperature, circadian rhythms, sexual behaviour, learning and memory, neuroendocrine function as well as neurotransmitters release. Some of the activities linked to NPY are summarized in Table 1.

	NPY biological actions	**Reference**
Feeding and drinking behaviours	Central injections of NPY increase food intake, even in satiated animals, and water consumption.	(Clark *et al.,* 1984)
	NPY stimulates food intake for hours when administered acutely, while chronically mimics hormonal and metabolic changes seen in obesity.	(Levine & Morley, 1984)
	Fasting increases NPY expression in the hypothalamic PVN.	(Kalra *et al.,* 1991)
	NPY is involved in the regulation of energy metabolism.	(Leibowitz ,1990)
Locomotor activity	Central NPY injection suppresses locomotion in rats both in their home cage and in the open field.	(Heilig & Murison, 1987)
	NPY increases locomotor	(Heilig *et al.* 1989b)

	activity in spontaneously hypertensive rats.	
	NPY injection into the frontal cortex increases locomotor behaviour in rats.	(Smialowski *et al.,* 1992)
Circadian rhythm	Microinjections of NPY in the suprachiasmatic nucleus cause a shift of the circadian rhythms.	(Albers & Ferris, 1984)
	NPY levels decrease during the light phase and decline thereafter, without NPY fluctuation in rats kept in complete darkness.	(Calza *et al.,* 1990)
Learning and memory	Post-training central administration of NPY improves memory retention in mice.	(Flood *et al.,* 1987)
	NPY can reverse amnesia induced by scopolamine.	(Flood *et al.,* 1987)
	NPY injection into the rostral hippocampus and septum enhances memory retention, while injected into the amygdala and caudal hippocampus induces amnesia.	(Flood & Morley, 1989)
	NPY enhances memory retention through inhibition of the release of GABA and vasoactive intestinal peptide.	(Morley & Flood, 1990)
Neuroendocrine regulation	NPY stimulates CRF mRNA in the hypothalamic PVN, both *in vivo* and *in vitro.*	(Tsagarakis *et al.,* 1989)
	Administered in the hypothalamic PVN, NPY increases ACTH, corticosterone and aldosterone in serum.	(Wahlestedt *et al.,* 1987)
	Depending on the hormonal state of the animals, NPY modulates release of luteinizing hormone from pituitary gland.	(McDonald *et al.,* 1985)
	Injected in the supraoptic nucleus, NPY enhances secretion of vasopressin.	(Willoughby & Blessing, 1987)
	Central NPY administration affects the secretion of growth hormone, prolactin and thyrotropin.	(Härfstrand et al., 1986)

Table 1. *Some of the central biological actions of NPY.*

1.5.2 Neuropetide Y receptors

The actions of NPY are mediated by specific NPY receptor subtypes that belong to the superfamily of the seven transmembrane domain G protein-coupled receptors and their activation leads to the inhibition of adenylate cyclase and the increase in intracellular calcium concentration (Hinson *et al.,* 1988). To date, seven mammalian receptor subtypes have been described, sorted into three distinct subfamilies, based upon their degree of amino acid sequence identity and named as their first members, i.e. Y_1, Y_2 and Y_5, which have been the most studied NPY receptors with respect to pharmacological profile and anatomical distribution (Wahlestedt *et al.,* 1986b; Herzog *et al.,* 1992; Larhammar *et al.,* 1992; Bard *et al.,* 1995; Rose *et al.,* 1995; Lundell *et al.,* 1995; Gerald *et al.,* 1995b; Gregor *et al.,* 1996; Weinberg *et al.,* 1996a). Their overall sequence identity is only 27-31% and the transmembrane domains share 40-43% identity (Larhammar *et al.,* 2001). The Y_1 subfamily includes the mammalian subtypes Y_1, Y_4 and Y_6 and they share approximately 50% overall amino acid identity which reaches 60% in the transmembrane regions (Larhammar *et al*., 2001). The Y_2 subfamily includes the Y_2 and the recently discovered Y_7 receptor, whereas the Y_5 receptor has not known relatives (Friedriksson et al., 2004).

1.5.2.1 NPY Y_1 receptor

The Y_1 receptor was the first NPY receptor cloned in the rat (Eva *et al.,* 1990), then the human clone was isolated (Larhammar *et al.,* 1992; Herzog *et al*., 1993) and it showed a 94% homology with the rat gene (Herzog *et al*., 1993). The Y_1 receptor is a seven transmembrane domain protein of 384 amino acids, G-protein bound, and it can couple to different second messengers, depending on the type of cells in which it is expressed. It has been showed that the Y_1 receptor activation is combined with inhibition of adenylyl cyclase in human cerebral cortex (Westlind-Danielsson *et al.,* 1987) and in SK-N-MC cells (Wahlestedt *et al.,* 1992a). In the rat CNS, the Y_1 receptor binding sites are concentrated postsynaptically in distinct layers of cerebral cortex, olfactory bulb, dentate gyrus of the hippocampus, amygdala, several thalamic and hypothalamic nuclei, island of Calleja and in the posterior part of the medial mammillary nucleus (Dumont *et al.,* 1996; Caberlotto *et al.,* 1997). However, in the human brain, the Y_1 receptor binding sites have been demonstrated to be predominantly localized in the dentate gyrus of hippocampus (Jacques *et al.,* 1997). The distribution of the Y_1 mRNA expression in the rat CNS does not correspond completely with the distribution of the Y_1 binding sites, for example at the level of hypothalamus (Larsen *et al.,* 1993;

Caberlotto *et al.,* 1998a); (Fig. 2). The Y_1 receptor seems to be involved in many physiological functions, such as modulation of locomotor activity (Heilig *et al.,* 1988), stimulation of food intake (Kanatani *et al.,* 1996), regulation of body weight and energy expenditure (Pedrazzini *et al.,* 1998) and central cardiovascular regulation (Yang *et al.,* 1993). Finally, Y_1 receptor has been proposed to be involved in seizures modulation, in view of the anticonvulsant properties of BIBP3226, a Y_1 specific antagonist (Gariboldi *et al.,* 1998).

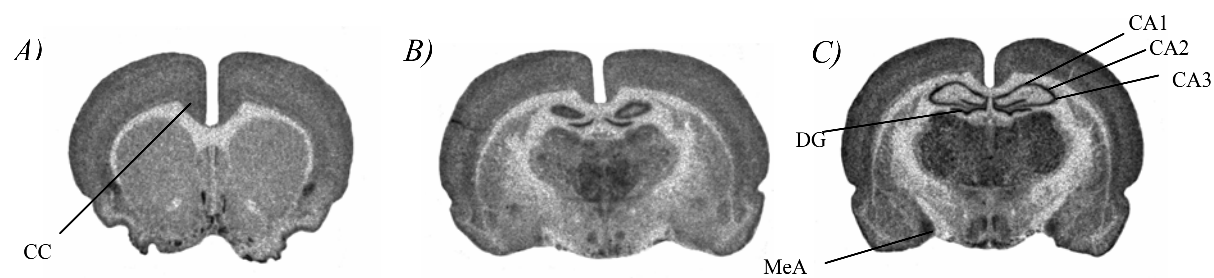


Fig. 2. *Hybridization signal of NPY Y_1 receptor mRNA expression in coronal sections of the rat brain, approximately at 1.60 mm (A), -1.88 mm (B) and -3.14 mm (C) from Bregma. Scale bar = 3.5 mm. CC, cingulate cortex; CA1, CA2, CA3, hippocampal CA1, CA2, CA3 regions; DG, dentate gyrus of hippocampus; MeA, medial amygdala.*

1.5.2.2 NPY Y_2 receptor

The Y_2 receptor is a protein consisting of 381 amino acids with a very low degree of homology (31%) with the Y_1 receptor. The Y_2 gene has been cloned in a variety of species, including human (Gerald *et al.,* 1995; Gehlert & Gackenheimer, 1997), and it presents a high degree of homology between different species: the rat and human receptors have 92% amino acids identity (St Pierre *et al.,* 1998). The Y_2 receptor, similar to the Y_1, is a seven transmembrane receptor, G-protein bound. The Y_2 receptors are coupled to the inhibition of adenylate cyclase (Wahlestedt *et al.,* 1992) and they decrease intracellular Ca^{2+} levels in a variety of neuronal preparations, with the exception of neuroblastoma cells, in which an increase of intracellular Ca^{2+} is observed (Ewald *et al.,* 1988; Bleakman *et al.,* 1992). In the rat CNS, the Y_2 specific binding sites have been found predominantly in the lateral septum, piriform cortex, bed nucleus of stria terminalis, dorsal hippocampus, ventral tegmental area, substantia nigra, dorsal raphe nucleus and cerebellum (Dumont *et al.,* 1996). The distribution of Y_2 mRNA expression is in line with the autoradiographical distribution (Gustafson *et al.,* 1997); (Fig. 3). The Y_2 receptor has been demonstrated to be the most abundant NPY

receptor subtype in the human brain, in particular in the hippocampal area (Widdowson, 1993; Jacques et al., 1997). This NPY receptor subtype is primarily located presynaptically, where it acts as an autoreceptor, inhibiting further release of NPY (Wahlestedt *et al.,* 1986; King *et al.,* 2000; Smith-White *et al.,* 2001). The Y_2 receptors have been implicated in a variety of NPY central functions: an activation of this receptor increases locomotor activity (Heilig *et al.,* 1989b), enhances memory retention (Flood and Morley, 1989), affects circadian rhythms (Golombek *et al.,* 1996) and mediates central cardiovascular functions (Aguirre *et al.,* 1990). In addition, Y_2 receptor subtypes can regulate the release of neurotransmitters: they suppress the release of noradrenaline in the locus coeruleus (Illes *et al.,* 1993) and glutamate in the hippocampus (Colmers *et al.,* 1991). Finally, the hippocampal Y_2 receptors appear to have an endogenous anticonvulsant activity, since they have been found to be increased in the hippocampus of an animal model of epilepsy (Schwarzer & Sperk, 1998; Schwarzer *et al.,* 1998).

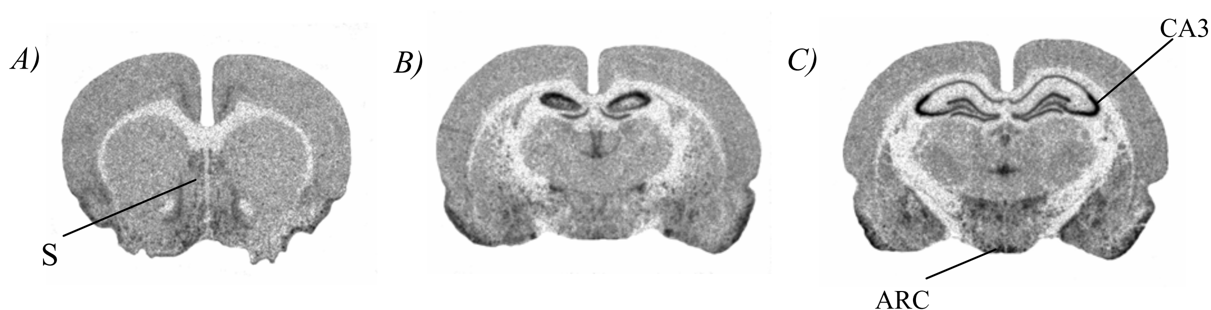


Fig. 3. *Distribution of NPY Y_2 receptor mRNA expression in coronal sections of the rat brain, approximately at 1.60 mm (A), -1.88 mm (B) and -3.14 mm (C) from Bregma. Scale bar = 3.5 mm. S, septum; CA3, hippocampal CA3 region; ARC, arcuate hypothalamic nucleus.*

1.5.2.3 NPY Y_3 receptor

The Y_3 receptor is the only NPY receptor subtype that has not been cloned yet, thereby very little information is available concerning the presence and distribution of Y_3 receptors in the CNS. Presence of putative Y_3 receptors has been described in the rat brainstem (Glaum *et al.,* 1997) and also in the hippocampal CA3 region (Monnet *et al.,* 1992). Some central functions have been ascribed to the Y_3 receptors, although the lack of selective compounds have put into question their effective role: it seems possible that central activation of Y_3 receptors, as well as Y_1 and Y_2 subtypes, could be associated with hypotension and bradycardia (Barraco *et al.,* 1990). Moreover, in adrenal chromaffin cells, Y_3 can inhibit the acetylcholine-induced release of catecholamine (Higuchi *et al.,* 1988).

1.5.2.4 NPY Y_4 receptor

The Y_4 receptor has been cloned in rat and human and it presents the lowest degree of homology (75%) between the two species. It is coupled to the inhibition of adenylate cyclase and mobilization of intracellular Ca^{2+} (Bard *et al.,* 1995). The autoradiographical distribution of Y_4 still remains to be established; however, using [^{125}I]bPP, Y_4 binding sites in the CNS of rats have been found in the interpeduncular nucleus, area postrema and nucleus of tractus solitarius (Whitcomb *et al.,* 1997), whereas using [^{125}I][Leu^{31}Pro^{34}]PYY, abundant Y_4 binding sites have not been found in the interpeduncular nucleus (Gehlert *et al.,* 1997).

1.5.2.5 NPY Y_5 receptor

The Y_5 receptor is a protein consisting of 445 amino acids and it was cloned by Hu and collaborators (1996). This receptor subtype has less than 35% amino acid identity with the other members of the NPY receptors family (Gerald *et al.,* 1996). The human protein is rather similar to the rat protein, presenting 87% overall amino acid identity. The Y_5 gene sequence has some overlap with the Y_1 gene, but the transcription of the Y_1 and the Y_5 genes occurs in opposite orientation (Gerald *et al.,* 1996). A shared promoter with the Y_1 has also been found, suggesting a possible co-regulation of Y_1 and Y_5 (Herzog *et al.,* 1997). The activation of Y_5 receptor causes an inhibition of adenylate cyclase activity (Gerald *et al.,* 1996). The Y_5 binding sites have been found in the olfactory bulb, lateral septum, anteroventral thalamic nuclei, CA3 region, nucleus tractus solitarius and area postrema, using [^{125}I][Leu^{31}Pro^{34}]PYY/BIBP3226-insensitive (Dumont *et al.,* 1998). The Y_5 mRNA expression is localized postsynaptically, mainly in the dentate gyrus and CA3 region of hippocampus, cingulate cortex and to a less extent in a number of hypothalamic nuclei (Gerald *et al.,* 1996); (Fig. 4). Regarding the functional role of Y_5 receptors, intracerebreventricular (i.c.v.) injections of Y_5 antisense oligodeoxynucleotides inhibit both NPY- and fasting-induced food intake in rats (Schaffhauser *et al.,* 1997). Moreover, CGP71683A, a non-peptide Y_5 receptor specific antagonist, inhibited NPY-induced food intake (Kask *et al.,* 2001). However, some studies and clinical evidences have questioned the role of Y_5 receptor in feeding behaviour (Small *et al.,* 1997; Kanatani *et al.,* 1997).

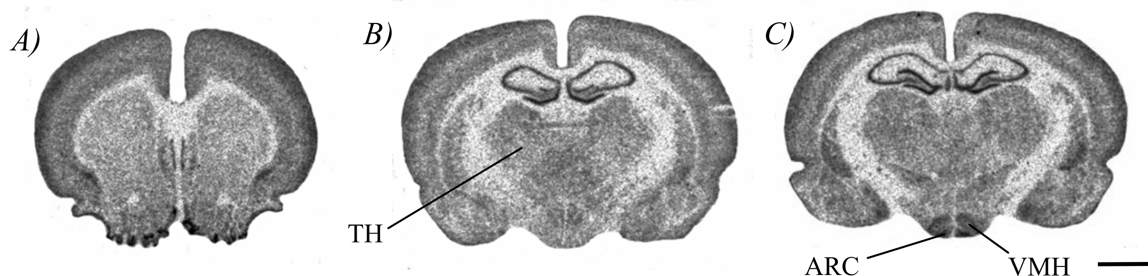


Fig. 4. *Representative images of the distribution of NPY Y_5 receptor mRNA expression in coronal sections of the rat brain, approximately at 1.60 mm (A), -2.12 mm (B) and -3.14 mm (C) from Bregma. Scale bar = 3.5 mm. TH, thalamus; VMH, ventral medial hypothalamic nucleus; ARC, arcuate nucleus of hypothalamus.*

1.5.2.6 NPY Y_6 receptor

The Y_6 gene appears not to be present in the rat brain, although it has been found in several other species, such as mouse and human, in which it is a pseudogene (Burkhoff *et al.,* 1998). The Y_6 receptor presents a very low homology to the other NPY receptors, but it share the highest identity (56% overall) with the Y_1 subtype (Weinberg *et al.,* 1996). Although Y_6 has the highest homology with the Y_1 receptor, the pharmacology is more similar to that of Y_2 (Weinberg *et al.,* 1996). In the murine CNS, a high expression of Y_6 receptor mRNA has been detected in the bed nucleus of the stria terminalis and in some hypothalamic nuclei, e.g. suprachiasmatic nucleus, anterior hypothalamus and ventromedial nucleus (Weinberg *et al.,* 1996).

1.5.2.7 NPY Y_7 receptor

The Y_7 receptor has been recently cloned from zebrafish (Friedriksson *et al.,* 2004). It has also been found in rainbow trout and in two amphibians, but this subtype seems to be lost in mammals. Its phylogenetic relationship to the other NPY receptors and its chromosomal position in the zebrafish genome suggest that Y_7, together with Y_2, constitute the Y_2 subfamily, namely origin from a common ancestor by chromosomal duplication. These two receptors present 50% homology in zebrafish, when comparing to the entire coding sequence. Despite this high level of homology, the pharmacological profile of the zebrafish Y_7 subtype differs considerably from mammalian Y_2, as it binds with low affinity endogenous NPY, PYY and the porcine peptides. It also binds short fragments of NPY, $NPY_{3\text{-}36}$ and $NPY_{13\text{-}36}$ with lower affinity, while the Y_2-specific antagonist BIIE0246 has showed no binding to the Y_7 receptor. The Y_7 mRNA has been primarily expressed in the gastrointestinal tract, eye and

some expression has been also found in the brain, whereas Y_2 receptor expression is mainly observed in the CNS (Friedriksson *et al.,* 2006, 2004).

1.5.3 NPY in neuropsychiatric disorders

In view of its distribution in the CNS and physiological functions, NPY has been widely investigated in relation to the mechanisms underlying the pathophysiology of a variety of neuropsychiatric illnesses, which are summarized in Table 2.

	Pathophysiological effects	Reference
Schizophrenia	Reduced PYY, but not NPY levels have been found in schizophrenics.	(Widerlov *et al.,* 1988)
	NPY-LI is higher in drug free schizophrenics compared to control.	(Peters *et al,.* 1990)
	NPY-LI is reduced in the temporal cortex of schizophrenics, but not in the hypothalamus and amygdala.	(Beal *et al.,* 1987b)
	Haloperidol withdrawal is linked to increased NPY-LI levels.	(Peters *et al.,* 1990)
Neurodegenerative disorders	In the Huntington's disease, NPY-LI is increased in the striatum, cerebral basal ganglia, substantia nigra, thalamic nuclei, bed nucleus stria terminalis, locus coeruleus and dentate nucleus of cerebellum.	(Dawbarn & Emson, 1985) (Beal *et al.,* 1988b)
	NPY is unchanged or decreased in the CSF of Alzheimer patients.	(Atack *et al.,* 1988; Alom *et al.,* 1990)
	Post- mortem studies reveal both unaltered and reduced NPY-LI in different cortical regions of Alzheimer patients.	(Gabriel *et al.,* 1993; Beal *et al.,* 1987a, 1986)
	Normal NPY concentration is reported in the striatum, while increased NPY and PYY are revealed in the hypothalamus of Alzheimer patients.	(Widerlöv *et al.,* 1991)
	NPY-positive neurons are diminished in the frontal and temporal cortices or unchanged in the globus pallidus and in the basal forebrain of Alzheimer's patients.	(Davies *et al.,* 1990)
	Cortical NPY levels are not altered or reduced in patients with severe Parkinson.	(Allen *et al.,* 1985; Beal *et al.,* 1988a)

	NPY levels are reduced in the CSF of patients with Parkinson's disease.	(Martignoni *et al.,* 1992)
Epilepsy	After seizures, NPY mRNA and binding sites are up-regulated in some cortical and limbic areas.	(Vezzani *et al.,* 1996 a,b)
	An anticonvulsant role of NPY is confirmed by *in vivo* studies.	(Smialowska *et al.,* 1996)
	NPY KO mice display spontaneous seizure and an induced sensitivity to pharmacologically induced convulsions, which are antagonized by centrally injected NPY.	(Erikson *et al.,* 1996)
Food-related disorders	NPY is released from terminals of the paraventricular hypothalamic nucleus of food restricted animals.	(Levenson, 2003)
	NPY mRNA increases in the hypothalamic arcuate nucleus of food-restricted and food-deprived male and female rats.	(Brady *et al.,* 1990)
	NPY concentration in the CSF of anorectic patients is significantly elevated.	(Kaye *et al.,* 1990)
	Plasma NPY levels in women with anorexia nervosa are reduced, while in women with bulimia nervosa are increased, compared to normal control subjects.	(Baranowska *et al.,* 2001)

Table 2. *Some of the pathophysiological changes observed in the neuropsychiatric disorders in which NPY is mainly involved.*

1.5.3.1 NPY and depression

A number of pre-clinical and clinical studies have proposed an involvement for NPY in the pathophysiology of depression and a role in the mechanism of action of antidepressant drugs (Widdowson *et al.* 1992). In the CNS, NPY is localized in limbic-related structures and in serotonergic and noradrenergic neurons (Everitt *et al.,* 1984; Halliday *et al.,* 1988; Blessing *et al.,* 1986). Interestingly, it has been showed that NPY can modulate the release of noradrenaline and serotonin, neurotransmitters involved in the pathophysiology of depression and the action of antidepressant drugs (Schlicker *et al.,* 1991; Finta *et al.,* 1992; Martire *et al.,* 1993). Clinical studies have suggested that the concentration of the NPY-LI in selected regions of post-mortem brains from suicide victims is reduced (Widdowson *et al.,* 1992). In particular, suicide victims with a previous history of depression have low levels of NPY-LI

in the frontal cortex and caudate putamen. Moreover, a decrease of NPY mRNA in the prefrontal cortex of subjects affected by bipolar disorder has also been described (Caberlotto & Hurd, 2001). In addition, decreased NPY-LI have been found in the cerebrospinal fluid (CSF) and plasma of depressed patients compared to healthy controls (Widerlov *et al.,* 1988; Hashimoto *et al.,* 1996; Nilsson *et al.,* 1996; Westrin *et al.,* 1999). In other investigations, however, no differences in NPY-LI in CSF or in post-mortem brain tissues have been observed between diagnostic groups (Irwin *et al.,* 1991; Ordway *et al.,* 1995). Additional data consistent with the NPY hypothesis are the findings of increased NPY-LI after antidepressant treatment or repeated ECT in depressed individuals (Mathé, 1996), suggesting that NPYergic hypofunction could play a role in the pathophysiology of depression. Likewise, animals treated with electroconvulsive stimuli (ECS), as well as lithium and antidepressants, show selective and specific effects on brain NPY-LI and NPY mRNA expression; in particular, ECS consistently increase NPY-LI and NPY mRNA in the hippocampal formation of rat (Mathé *et al.,* 1990; Wahlestedt *et al.,* 1990; Stenfors *et al.,* 1992, 1989). The observed changes have suggested that antidepressants may exert some of their therapeutic effects through the up-regulation of the endogenous NPY. Other studies, however, failed to demonstrate any alteration of the NPY system following antidepressant treatment (Heilig & Ekman, 1995; Bellmann & Sperk, 1993). The NPY system has also been largely studied in animal models of depression and some of the observed changes are summarized in Table 3.

Animal Model	Effect	Reference
Flinders Sensitive Line rats	↑↓ NPY mRNA ↑↓ NPY immunoreactivity ↓ NPY immunoreactivity	(Caberlotto *et al.,* 1998a) (Caberlotto *et al.,* 1999) (Jimenez-Vasquez *et al.,* 2000)
Fawn Hooded rats	↓ NPY immunoreactivity	(Mathé *et al.,* 1998)
Maternal separation	↓ NPY immunoreactivity	(Jimenez-Vasquez *et al.,* 2001)
Olfactory bulbectomy	↑ NPY gene expression ↑ NPY immunoreactivity	(Holmes *et al.,* 1998; Primeaux & Holmes, 2000)
Social isolation	↑ ↔ NPY levels	(Thorsell *et al.,* 2005)

Table 3. *Summary of the pre-clinical evidences indicating a role for NPY in depression.* ↑: increase, ↓: decrease, ↑↓: increase/decrease depending on brain region, ↔: no change.

1.5.3.2 NPY and anxiety

Experimental studies have demonstrated an anxiolytic-like effect in rodents after NPY i.c.v. administration or into the central amygdala (Heilig *et al.,* 1992). In addition, behavioural

studies have also suggested the anxiolytic and sedative action of the peptide (Heilig & Murison, 1987). In contrast, transgenic mice with brain overexpression of NPY have displayed an anxiety-like behaviour (Inui *et al.,* 1998).

Less is known about the implication of NPY in human anxiety disorders. A clinical study has showed a trend for a negative correlation between NPY CSF levels and anxiety levels obtained using the Hamilton Depression rating scale in patient diagnosed with major depression (Widerlow *et al.,* 1989).

1.5.4 NPY receptors in depression and anxiety

1.5.4.1 NPY Y_1 receptor in depression and anxiety

Numerous pre-clinical evidences have supported a role for NPY and the Y_1 receptor in the pathophysiology of depression and anxiety disorders, evidencing that this receptor subtype generally mimics the actions of NPY (Dumont *et al.,* 1996; Caberlotto *et al.,* 1997).

The Y_1 receptor mRNA expression and binding sites have reported significant changes in specific brain regions of the FSL rats, an animal model of depression, suggesting that this receptor subtype plays an important role in the mechanisms underlying the pathophysiology of depression (Caberlotto *et al.,* 1998a; Caberlotto *et al.,* 1999; Zambello *et al.,* 2007; Jimenez-Vasquez *et al.,* 2006; Jimenez-Vasquez *et al.,* 2007). Moreover, chronic antidepressant treatments have showed to increase the Y_1 receptor subtype mRNA levels in certain brain regions on the FSL rats (Caberlotto *et al.,* 1998a). An hippocampal coadministration of NPY and BIBO3304, a Y_1 receptor selective antagonist, has blocked the antidepressant-like effect of NPY in the learned helplessness rats, an animal model of depression, while an injection of the Y_1 and Y_5 preferring agonist [$Leu^{31}Pro^{34}$]PYY into the same region has produced an antidepressant-like effect (Ishida *et al.,* 2007). Moreover, an i.c.v. injection of NPY and the Y_1 preferring agonist [$Leu^{31}Pro^{34}$]PYY has reduced the immobility time in the mouse forced swim test (FST) in a dose-dependent manner, thus inducing an antidepressant effect, which was blocked by the injection of the Y_1 antagonists BIBP3226 and BIBO3304, obtaining a depressive-like effect (Redrobe *et al.,* 2002). Furthermore, it has been found that the injection in the amygdala of BIBO3304 produced an anxiolytic-like effect in the social interaction test (Sajdyk *et al.,* 1999) and in the elevated plus maze (EPM) in rats (Primeaux *et al.,* 2005). However, an anxiogenic-like effect of the centrally administered Y_1 receptor antagonist BIBP3226 has been demonstrated in the EPM

(Kask *et al.,* 1996) and in the social interaction test in rats, after administration in the dorsal periacqueductal gray matter (Kask *et al.,* 2002, 1998b). In agreement with these findings, a role for the Y_1 receptor in the anti-anxiety effect of NPY has been proposed since injections of a Y_1 antisense oligonucleotide in amygdala caused an anxiety-like state in the rat (Heilig, 1995). Furthermore, an anxiolytic-like activity of the Y_1 receptor agonists [$Leu^{31}Pro^{34}$]NPY and [$Gly^{6}Glu^{26}Lys^{26}Pro^{34}$]NPY has been observed in the conflict test (Britton *et al.,* 1997). Moreover, the Y_1 receptor agonists have showed anxiolytic-like effects both in the EPM and open field tests of anxiety (Sorensen *et al.,* 2004).

Studies conducted on mice lacking the Y_1 receptor have suggested that the Y_1 deficiency resulted in marked alterations in the anxiety-related behaviours in the open-field, EPM and light-dark tests (Karl *et al.,* 2006; Karlsson *et al.,* 2008), assessing that the Y_1 receptor has a prominent role in the anxiolytic-like effects of NPY.

At present, the information regarding the possible alteration of the NPY receptors in human subjects diagnosed with affective disorders or other psychiatric disorders is still reduced. The Y_1 receptor mRNA expression has been investigated in the prefrontal cortex of post-mortem depressed patients, but no changes have been reported (Caberlotto & Hurd, 2001).

1.5.4.2 NPY Y_2 receptor in depression and anxiety

Numerous pre-clinical studies have described a role for the Y_2 receptor in the pathophysiology of depression and anxiety, assessing that this receptor subtype could regulate NPY release, probably for its presynaptical location.

A chronic antidepressant treatment has reduced the NPY binding to the Y_2 receptor subtype in discrete rat brain regions (Widdowson & Halaris, 1991). However, a previous study on the FSL rats, an animal model of depression, has failed in demonstrating a role for the Y_2 receptor in the mechanisms underlying the pathophysiology of depression, since alterations of the Y_2 mRNA expression have not been found (Caberlotto *et al.,* 1998a). Contrasting data are available on the involvement of Y_2 receptors in anxiety, depending on the brain site or receptor ligand injection and the type of behaviour tested. An anxiogenic-like effect of NPY through the Y_2 receptor has been proposed, in view of the effect of the i.c.v. injection of $NPY_{13\text{-}36}$, the truncated form of NPY, in mice tested in the EPM behavioural paradigm (Nakajima *et al.,* 1998); however, no effect of NPY on punished responses has been evaluated in the rat conflict tests (Heilig *et al.,* 1989; Britton *et al.,* 1997). Moreover, intra-amygdaloid injections of Y_2 receptor agonist have increased anxiety (Sajdyk

et al., 2002), whereas injections close to the locus coeruleus have resulted in an anxiolytic-like response (Kask *et al.,* 1998b; Sajdyk *et al.,* 2002) in the social interaction test and in the EPM, respectively. Finally, an anxiolytic-like profile of BIIE0246, a Y_2 receptor antagonist, has been observed in the rats after exposure to the EPM (Bacchi *et al.,* 2006).

Numerous studies have investigated the depressive- and anxiety-related behaviours in the Y_2 knockout (KO) mice, which have demonstrated reduced immobility in the FST, supporting a role for the Y_2 receptor in the antidepressant-like behaviour (Tschenett *et al.,* 2003; Carvajal *et al.,* 2006), and increased time spent in the open arms of the EPM, demonstrating an involvement of the Y_2 receptor in the modulation of an anxiolytic-like profile (Redrobe *et al.,* 2003; Tschenett *et al.,* 2003).

Finally, a post-mortem study conducted on human pre-frontal cortex of depressed patients has failed in finding any changes of the Y_2 receptor mRNA expression levels compared to the controls (Caberlotto and Hurd, 2001).

1.5.4.3 NPY Y_5 receptor in depression and anxiety

Although the Y_5 receptor has been mostly studied for its involvement in the mechanisms related to food intake and obesity (Gehlert, 1999; Cabrele & Beck-Sickinger, 2000), in view of its distribution in brain regions known for their role in the emotional disorders, some recent studies have also assigned a role for this receptor subtype the emotional responses to stress and in mediating the anxiolytic-like effects of NPY. In fact, it has been described to mediate the anxiety-like state in the basolateral amygdala in the social interaction test (Sajdyk *et al.,* 2002); moreover, specific Y_5 receptor agonists have showed an anxiolytic-like activity in the EPM and open field test of anxiety (Sorensen *et al.,* 2004). However, contrasting data have been presented in a study involving the Y_5 receptor antagonist CGP71683A; in fact, it has failed in demonstrating an involvement for this receptor subtype in mediating the NPY-induced anxiolysis in the rat social interaction test, EPM and open field (Kask *et al.,* 2001).

2. AIMS

The major objectives of this study consisted on improving the knowledge on the involvement of the NPY system in mood disorders, in particular depression and anxiety, investigating the role of the different NPY receptor subtypes in the modulation of the NPY function in mood disorders. In particular, the purpose of the present study was to investigate which of the three NPY major receptor subtypes Y_1, Y_2 and Y_5 has a principal role in regulating the NPY functions in mood disorders, given that the lack of selective, brain penetrant, specific antagonists has created difficulties in establishing which one could be the mainly involved.
The specific aims of this study were to:

- Perform a behavioural characterization of the NPY Y_2 KO mice, giving further support to some previous evidences reporting that the Y_2 KO mice have displayed reduced anxiety and increased stress coping ability. Subsequently, the role of the Y_1 receptor would be investigated in these mice, to assess if a possible compensatory mechanism involving Y_1 could be activated in mice lacking the Y_2 receptor.
- Analyze the possible alterations of the NPY system mRNA expression in three different animal models of depression: a genetic model - the FSL rats - and two chronic stress models - the chronic mild stressed rats and the chronic social defeated rats and tree shrews - to possibly define the receptor subtype with a major role and the brain regions mainly involved in the affective disorders. Finally, the existance of species-specific differences in the expression and functions of the NPY system would be considered.
- Investigate the possible alterations of the Y_2 receptor mRNA expression in human post-mortem amygdala of psychiatric subjects affected by major depression, bipolar disorder and schizophrenia, compared to normal controls, to detect if potential alterations of the Y_2 receptor mRNA in this region known for its involvement in the regulation of affective behaviour and neurochemical responses to stress could be directly associated with these neuropsychiatric diseases.

3. MATERIAL AND METHODS

3.1 CHARACTERIZATION OF THE NPY Y_2 KO MICE

3.1.1 In vivo experiments

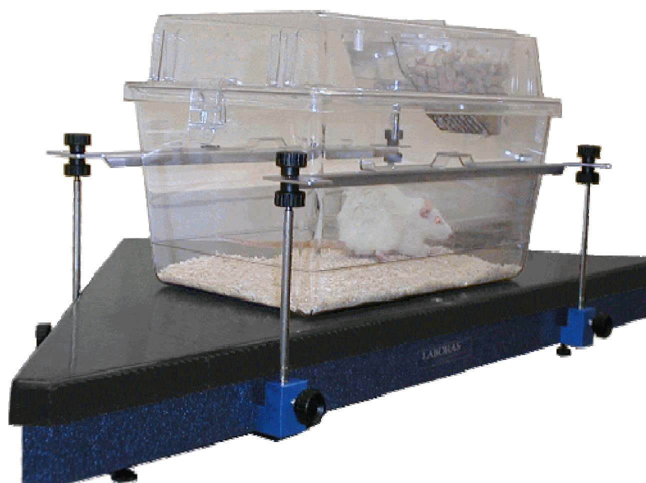
The strategy used to generate the Y_2 KO mice consisted on using the cre/loxP technology to delete the entire coding sequence of the Y_2 receptor on mixed 129SvJ-C57BL/6 genetic background mice (Sainsbury *et al.,* 2002). These mice were generated at the Garvan Institute of Medical Research, Sydney, Australia. Successively, they were sent to GlaxoSmithKline, where the mixed genetic background mice were back-crossed for seven generations with the pure C57BL/6 genetic background mice, to obtain Y_2 KO mice with a pure C57BL/6 genetic background.

In the present study, 9-12 weeks old male and female Y_2 KO mice and their controls wild type (WT) mice were used. They were kept under standard 12 hours light (6:00 a.m. – 6:00 p.m.) and 12 hours dark (6:00 p.m. – 6:00 a.m.) cycles and fed a standard chow diet and tap water *ad libitum*. Male and female mice were individually caged in two separated rooms., Mice were allowed to habituate to the testing room at least one hour before all the behavioural tests. All the tests were carried out between 09:00 a.m. and 3:00 p.m. Test environments were thoroughly cleaned between test sessions.

All the procedures involving the care of the animals and the experimental protocols were conducted in conformity with the institutional guidelines, in compliance with national and international laws and policies.

3.1.1.1 LABORAS system

The Laboratory Animal Behaviour Observation Registration and Analysis System (LABORAS^{TM}) system consisted of a triangular shaped sensing platform (700 x 1000 x 30 mm) positioned on two orthogonally placed force transducers. The whole structure stood on three spikes, adjustable in height, and adsorbed external vibrations. One cage

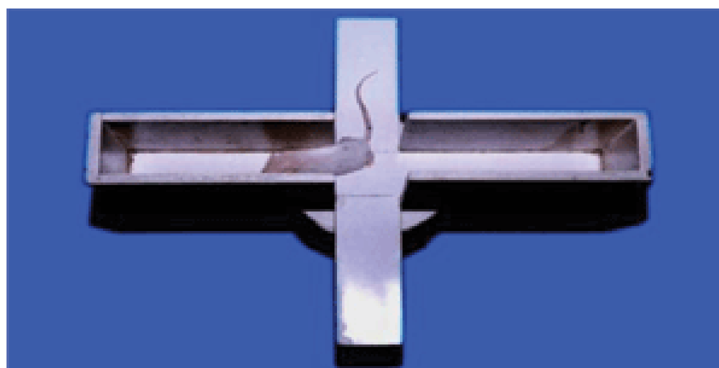


was placed directly onto the sensing platform, the upper part of which (including the top, food hopper and drinking bottle) was suspended in a high adjustable frame and was free from the sensing platform. The resultant electrical signals caused by the mechanical vibrations of the movement of the animal were transformed by each force transducer, amplified to a fixed signal range, filtered to eliminate noise, digitized and stored on a computer. The computer then processed the stored data using several signal analysis techniques to classify the signals into the behavioural categories of feeding, drinking, climbing, grooming, locomotor activity and total distance.

The Y_2 KO and WT male (n = 15-16) and female (n = 8) mice were habituated to the LABORAS cage two hours before the beginning of the recording, which was then performed for 24 hours.

3.1.1.2 Elevated Plus Maze (EPM)

The EPM is a pharmacologically validated test of anxiety (Pellow & File, 1986). The device consisted of a central part (5 x 5 cm), two opposing open arms (30 x 5 cm) and two opposing closed arms of the same size, with 14 cm high, non transparent walls. The maze was elevated 50 cm above the floor and exposed to a red dim light. At the beginning of each trial, mice were placed on the central platform facing an open arm. Mice were tested randomly and their behaviour was videotaped and subsequently analyzed by an observer blind to the genotype. Entry into an arm was defined when the mouse placed its four paws in that arm. The number of entries into the open and closed arms and the time spent in each type of arm were determined.



The test was performed for 5 minutes on Y_2 KO and WT male mice (n = 9-10); among them, some had been previously exposed to 5 minutes of restraint stress, by placing them in a plexiglass tube.

3.1.1.3 Forced Swim Test (FST)

The FST is a pharmacologically validated test to examine the depressive-like state of the animals (Porsolt *et al.,* 1977). In the present study, the FST was performed in two different modalities: the first one was conducted on the same male mice (n = 8) tested 10 days before

with the LABORAS system. Each mouse was individually placed in an open container (diameter 10 cm, height 25 cm), filled with 10 cm deep tap water maintained at 25ºC. Its activity was videotaped over a period of 6 minutes and the total time of immobility was measured during the last 4 minutes by an observer blind to the genotype. The second modality was applied both on male and female mice (n = 8) previously exposed to the LABORAS and EPM tests, which were placed in a container (diameter 18 cm, height 35 cm) filled with 15 cm deep tap water maintained at 25ºC. Their activity was videotaped for a period of 10 minutes and the immobility time was considered during the last 5 minutes. Mice were considered immobile when floating passively in the water, performing only those movements required for keeping their heads above the water level.

3.1.1.4 FST after desipramine treatment

The FST was also performed on Y_2 KO and WT male (n = 8) and female (n = 10-11) mice, previously intraperitoneally (i.p.) administered with two different doses (10 mg/Kg and 20 mg/Kg) of the antidepressant desipramine, whereas the control animals were injected i.p. with vehicle (saline solution). The body weight of the male mice was measured between 23 g and 33 g and the body weight of the female mice was measured between 14 g and 22 g. The injections were performed 30 minutes before the beginning of the behavioural tests, occurred using a container (18 cm diameter, 35 cm height) filled with 15 cm deep tap water at 25ºC. The FST was conducted over a period of 10 minutes and the immobility time was considered between the second and the sixth minute.

3.1.1.5 ACTH and corticosterone levels after FST at 21°C

The FST conducted using a container (diameter 10 cm, height 25 cm) filled with 10 cm deep tap water maintained at 21ºC was considered a kind of stress and it was performed only on male mice (n = 16). They were individually placed in the container for 5 minutes, while the animals not exposed to stress (n = 16) were maintained into their home cages. Five minutes after the end of the stress procedure, control and stressed mice were all sacrificed by decapitation. From each animal, a blood sample was collected and it was stored either in a container with K^+- EDTA, to prevent the coagulation, obtaining the plasma, or in a container without this substance, obtaining the serum. Each sample was then centrifuged at 1800 rcf speed for 10-15 minutes at 4ºC; subsequently, the plasma ACTH levels and serum

corticosterone levels were measured using specific radioimmunoassay kits, named respectively IRMA (Immunoradiometric Assay, DiaSorin) and RIA (Radioimmuno Assay, MP Biomedicals).

3.1.1.6 Statistical analysis

The statistical analysis was performed using the "Statistica 6.0" software package for the analysis of variance. The data obtained from the LABORAS, FST and EPM studies were subjected to a paired Student's t-test, comparing the two groups of mice, Y_2 KO and WT. In the FST, the effect of the desipramine treatment *vs* vehicle on the immobility time in the Y_2 KO and WT mice was assessed by the analysis of variance (one-way ANOVA), followed by the Duncan's *post hoc* test when appropriate. Finally, a two-way ANOVA was carried out to evaluate the effect of stress (FST at 21ºC) on the ACTH and corticosterone levels in the two groups of animals.

All the results were expressed as mean ± S.E.M. and in all the statistical analysis the p-value has been considered significant if lower than 0.05.

3.1.2 In vitro experiments

The data obtained with the behavioural experiments were supported by a series of *in vitro* analysis, to confirm the deletion or presence of the Y_2 receptors in the Y_2 KO mice and in their controls WT mice.

3.1.2.1 Reverse Transcriptase-PCR reaction (RT-PCR)

To demonstrate the central deletion of the Y_2 receptor in the Y_2 KO mice and its presence in the WT mice, a RT-PCR was conducted as follows:

- Tissue collection and total RNA extraction: since the Y_2 receptor has been demonstrated to be highly expressed in the hippocampus (Dumont *et al.,* 1996), this region was dissected out from the brains of three Y_2 KO and three WT mice, collected in 200 µl of RNAlater solution (Qiagen) and kept at 4°C overnight. The day after, the RNAlater solution was removed and the samples were frozen in dry ice and stored at -80ºC until processed. The tissues were homogenized with a rotor-stator homogenizer. After the homogenization was complete, total RNA was isolated using the RNeasy Mini kit (Qiagen), following the manufacturer's protocol. Potential trace amounts of residual genomic DNA were removed with the addition of RNase-free DNase I (Qiagen). Purified total RNA samples were eluted in RNase-free water and stored at -80°C. Quality control evaluation of RNA samples was

performed with the Agilent 2100 Bioanalyzer (Agilent Technologies).

• complementary DNA (cDNA) synthesis: a reverse transcription reaction was then performed using the First-Strand cDNA Synthesis kit (invitrogen), in which SuperScript II reverse transcriptase reacted for 50 minutes at 42ºC to convert total RNAs into single stranded cDNAs. For each sample, triplicate reverse transcription reactions (RT+) were performed, while an additional reaction in which the enzyme had been omitted (RT-) was carried out to exclude the possibility of an eventual genomic DNA contamination.

• DNA oligonucleotides primers selection and RT-PCR: the RT-PCR reaction was conducted using four couples of oligonucleotides primers, selected from the mRNA sequence of the *mus musculus* Y_2 receptor (NM_008731) contained in the GenBank database (http://www.ncbi.nlm.nih.gov/). The primers were designed using the Primer Express v1.00 software (Applied Biosystems) and they were the followings (forward and reverse, obtained product length and annealing temperature):

1) 5'-ctt tag agg tcc acc gag aa-3' and 5'-ggg ctc cac ttt cac ttc ta-3'; 303 base pairs (bp); 56ºC;
2) 5'- act gct cca tca tct tgc ta-3' and 5'- tct cca ggt ggt aga caa tg-3'; 303 bp; 56ºC;
3) 5'-aga cct ccc att gta ttg act c-3' and 5'-ccg aaa cat tact cgt ata gca-3'; 489 bp; 56ºC;
4) 5'-tga gag caa aca aag ttc aca-3' and 5'-ttc aac gat tca ctt cag aca-3'; 801 bp; 54ºC.

All the amplification reactions were performed in a total reaction volume of 30 µl, composed by 300 nM of each primer, 200 µM dNTPs, 1.5 mM 10X buffer, 2 mM $MgCl_2$, 2.5 unit/reaction Hot Star Taq DNA polymerase (Qiagen), 10 ng cDNA and sterile water. Using the first three couples of primers, the RT-PCR reaction protocol was the following: 15 minutes at 95ºC, 35 cycles at: 94ºC for 30 seconds, 1 minute at 56ºC and 1 minute at 72ºC, plus 7 additional minutes at 72ºC. Using the fourth couple of primers, the protocol applied was the same, but the annealing temperature was 54ºC. As negative controls, a RT-PCR reaction performed without the cDNA as a template was conducted for each sample. The RT-PCR products were then visualized through a 0.7% agarose gel electroforesis.

3.1.2.2 Receptor autoradiography

To visualize the Y_2 receptor binding sites in the WT mice and to demonstrate their absence in the Y_2 KO mice, a receptor autoradiography was performed using [^{125}I]PYY radioligand, which binds indistinctively all the NPY receptors (Gobbi *et al.,* 1999). As previously mentioned, the Y_2 receptor binding sites are abundant in many different brain areas, but mainly in the lateral septum, piriform cortex, bed nucleus stria terminalis, hippocampal

formation, ventral tegmental area, substantia nigra, dorsal raphe nucleus and cerebellum (Dumont *et al.,* 1996). The receptor autoradiography was performed on coronal sections (14 µm-thick) cut from a Y_2 KO and a WT mouse, approximately at -1.34 mm from Bregma (Paxinos and Watson, The Mouse Brain in Stereotaxic Coordinates, 1997, Academic Press). The sections were preincubated in 1X HEPES buffer for 30 minutes at room temperature (10X HEPES buffer was made with 137 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 1.26 mM $CaCl_2$, 0.81 mM $MgSO_4$, 20 mM HEPES, 0.3% BSA, pH 7.4). Successively, the sections were incubated in the same buffer containing 25 pM [^{125}I]PYY (2200 Ci/mmol, Amersham) alone (total binding, T.B.) or added with 1 µM unlabelled NPY (non specific binding, N.S.B.) for 60 minutes at room temperature. The sections were subsequently washed four times (2 minutes each) in ice-cold Tris-HCl 50 mM, pH 7.4, briefly dipped in deionized water, quickly dried in a stream of cold air and then exposed overnight to a BAS-IP-SR 2025 Fuji photo film. The autoradiogram was then visualized using an image analysis software system (AIS 4.0, Imaging Research).

3.1.2.3 In situ hybridization

The experiment was performed on coronal sections (14 µm-thick) cut from two WT and two Y_2 KO mice (two slices for each animal, at -1.34 mm from Bregma; Paxinos and Watson, The Mouse Brain in Stereotaxic Coordinates, 1997, Academic Press) using a rat Y_2 receptor riboprobe (95% sequence homology with mouse mRNA sequence). The procedures of the riboprobe preparation and *in situ* hybridization reaction will be described in detail in the sections 3.2.2.1 and 3.2.2.2. After the experimental procedure, the slides were exposed to a Fuji Imaging plate (BAS-TR 2025) together with ^{14}C standards for 4-5 days. The images obtained from the *in situ* hybridization experiment were not exposed to a quantitative analysis, but only to a qualitative inspection.

3.1.2.4 Real-time quantitative PCR reaction (RT-qPCR)

The RT-qPCR or TaqMan analysis, one of the most precise and sensitive methods for the quantification of gene expression (Schmittgen *et al.,* 2000), was performed to verify the presence of eventual differences between C57BL/6 and 129SvJ mice in the basal hypothalamic NPY levels.

• Tissue collection, total RNA extraction and cDNA synthesis: five C57BL/6 and five 129SvJ male mice were sacrificed by head dislocation. Their brains were quickly removed under RNAse-free conditions and the entire hypothalamic regions were dissected out,

collected in RNAlater solution (Qiagen) and kept overnight at 4ºC. The total RNAs were then isolated, purified and converted into single stranded cDNAs, as previously described (section 3.1.2.1). The final products were directly used for TaqMan analysis.

• TaqMan DNA oligonucleotides primers and probes: TaqMan primers and probe sequences specific for mouse NPY and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) gene, used as control housekeeping gene, are listed in the Table 4 reported below. All the DNA oligonucleotide primers were custom synthesized by Proligo Europe.

The NPY primers were designed from a public sequence contained in the GenBank database (http://www.ncbi.nlm.nih.gov/) using Primer Express v.1.00 software (Applied Biosystems). The amplicon size was 74 bp for NPY and the selected annealing temperature was 60°C. The primer pairs were selected to amplify a sequence close to the 3' coding region of the target gene and to keep each primer pair within the same exon, in order to be able to use mouse genomic DNA for the standard curve, obtained for each primer pair by five serial decimal dilutions of mouse genomic DNA. In addition, the absence of genomic DNA was tested in cDNA samples prepared in the reverse transcription reactions in which the enzyme was omitted (RT-). The primers and probe sequences for mouse GAPDH gene were obtained from P. Murdock (Quantitative Expression Dept. GlaxoSmithKline, Stevenage, UK). The amplicon size was 90 bp for GAPDH and the annealing temperature was 60°C.

• RT-qPCR assay: the RT-qPCR reactions were performed in triplicate in 96-well optical plates (Applied Biosystems) in 30 µl per well, each containing 2X TaqMan® Universal PCR Master Mix kit (Applied Biosystems), forward and reverse primers (300 nM each), TaqMan probe (6 µM) and 5 µl cDNA template. The amount of total cDNA amplified was related to the mRNA abundance of the target gene; generally, the totally used material was 20 ng. On each plate, three reactions were performed without adding cDNA template, but water, as negative controls; then three replicates of each dilution of the standard curve and three replicates for each cDNA preparation were added. The plates were analyzed with the ABI PRISM 7900HT Fast sequence detector (Applied Biosystems). Cycling parameters were: two initial steps at 50°C for 2 minutes and at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. Data were acquired and processed with the SDS software v2.3 (Applied Biosystems). The quantity of PCR products was monitored by measuring the increase in fluorescence; these measurements resulted in an amplification plot of the fluorescence signal *vs* cycle number. The parameter Ct (threshold cycle) was defined as the fractional cycle number at which the fluorescence passes a fixed threshold, automatically set by the software at 10 standard deviations above mean

fluorescence generated during baseline cycles (from 3 to 15). The quantification of the unknown samples was calculated from their Ct values by interpolation from the standard curve to yield a relative gene expression measure. After the final cycle of the RT-qPCR, primer specificity was checked by the dissociation curve method, according to the Applied Biosystems protocol. Heat dissociation of the amplified DNA detected a single peak, thus showing that a single, specific PCR product was synthesized.

• Statistical analysis: data were analyzed with the analysis of covariance (ANCOVA) for statistical significance ($p < 0.05$), followed by Dunnett's post-hoc test, as previously described (Bond *et al.,* 2002). The average quantity relative to total RNA input was calculated for each sample. The expression data for the housekeeping gene GAPDH were used as covariate, in order to remove the effects due to RNA and cDNA samples quality from the analysis. The covariance efficiency factor measured the probability that the treatment affected the expression of the selected housekeeping gene (Bond *et al.,* 2002).

Mouse target gene	**Sequence**	**Tm [°C]**
GAPDH forward	CAAGGTCATCCATGACAACTTTG	61
GAPDH reverse	GGGCCATCCACAGTCTTCTG	63
GAPDH probe	ACCACAGTCCATGCCATCACTGCCA	69
GAPDH standard	GGGGCCATCCACAGTCTTCTGAGTGGCAGTGATGGCATGGACTGTGGTCATGAGCCCTTCCACGATGCCAAAGTTGTCATGGATGACCTTG	-
NPY forward	TTTCCAAGTTTCCACCCTCATC	59
NPY reverse	AGTGGTGGCATGCATTGGT	58
NPY probe	ATCTCATCCCCTGAAACCAGTCTGCCTG	70
NPY standard	CTAGTGGTGGCATGCATTGGTGGGACAGGCAGACTGGTTTCAGGGGATGAGATGAGATGAGGGTGGAAACTTGGAAAA	-

Table 4. *List of forward and reverse primers, probes and standards used in the TaqMan analysis. Their melting temperatures were reported, when available.*

3.2 ANIMAL MODELS OF DEPRESSION

3.2.1 Behavioural protocols and tissues collections

3.2.1.1 Flinders Sensitive Line rats

The behavioural experiments on the Flinders rats were performed at the Karolinska Institute, Stockholm, Sweden. Animals were treated in accordance with protocols approved by the Animal Ethical Committee of Stockholm and all the experimental procedures were conducted in conformity with the Karolinska Institutet's Guidelines. Efforts were made to minimize the number of animals used and to reduce their sufferings.

Twelve FSL and twelve FRL adult male rats weighing 240-260 g at the beginning of the study were kept under standardized light conditions at a constant room temperature of 23˚C, with free access to food pellets and tap water. Six FSL and six FRL rats were subjected to one hour acute restraint, by placing them in a plexiglass tube, and after the stress procedure, they were returned in their home cages. The control rats were left in their home cages and handled without exposure to the stress procedure. All the animals were sacrificed four hours after the end of the exposure to stress, based on a previous study reporting NPY mRNA changes in the arcuate hypothalamic nucleus following restraint (Makino *et al.,* 2000). Their brains were then removed, immediately frozen by immersion in isopentane, then stored at -80˚C and shipped to GlaxoSmithKline laboratories for the *in vitro* experiments. Coronal sections (14 µm-thick) were cut from the entire brains using a CM3050S cryostat (Leica), approximately at 1.60 mm, -1.88 mm, -2.30 mm and -3.14 mm from Bregma (Paxinos and Watson, The Rat Brain in stereotaxic coordinates, 1998, Academic Press), thaw-mounted onto polarized SuperFrost Plus slides and then stored at -80˚C until usage in the *in situ* hybridization experiments.

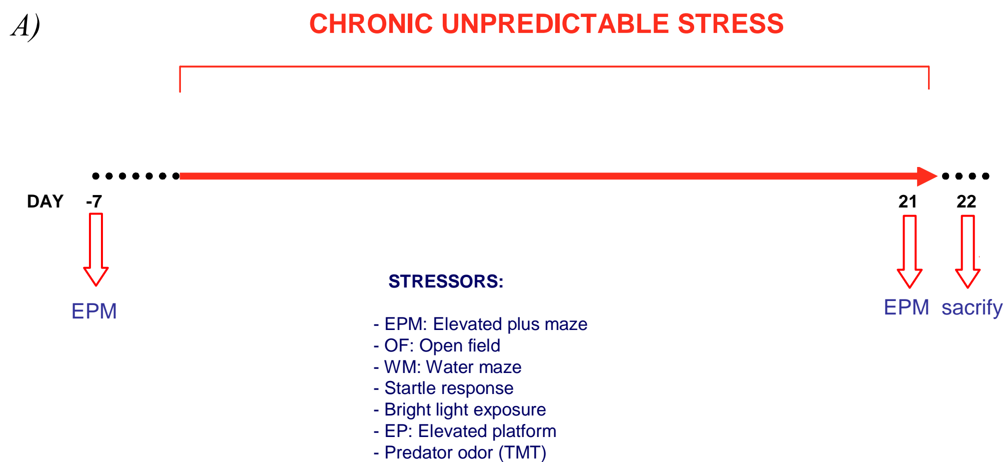
3.2.1.2 Chronic mild/unpredictable stress

The chronic mild stress paradigm was performed at the Laboratoire de Génétique Comportementale, Brain and Mind Institute, Ecòle Polytechnique Fédérale de Lausanne (EPFL), Switzerland. All the procedures involving the care of the animals and the experimental protocols were conducted in conformity with the institutional guidelines, in compliance with Switzerland national rules and international laws and policies.

Twenty Sprague-Dawley rats, weighing 250 g at the beginning of the study, were kept under standardized light conditions at a constant room temperature of 22˚C, with free

access to food pellets and tap water. Seven days before the beginning of the CMS protocol, all the rats were exposed to an EPM test, used as selective criterion to define two groups of animals: "high anxiety" (HA; n = 7) and "low anxiety" (LA; n = 5) rats, based on the time they spent in the open arms of the maze and on the number of entries in the open arms, representative of the anxiety levels of the animals (Landgraf & Wigger, 2002). Among them, some rats were subsequently exposed to a CMS protocol for 21 days, always during the light phase, but at different times of the day, while the non stressed animals were left in their home cages and exposed to daily handling. Each stress procedure was conducted in a different experimental room, also different from the room in which the animals were stored. Each kind of stress and the experimental modalities are summarized in Fig. 5. At the end of the CMS protocol, the rats were exposed to another EPM to evaluate their final anxiety level and 24 hours after this last stress all the animals were sacrificed. The brains were removed and immediately frozen by immersion in isopentane, stored at -80˚C and shipped to GlaxoSmithKline laboratories for the *in vitro* experiments. Coronal sections (14 µm-thick) were cut from the entire brains using a CM3050S cryostat (Leica), approximately at 1.60 mm, -1.88 mm, -2.30 mm and -3.14 mm from Bregma (Paxinos and Watson, The Rat Brain in stereotaxic coordinates, 1998, Academic Press), thaw-mounted onto polarized SuperFrost Plus slides and then stored at -80˚C until usage in the *in situ* hybridization experiments.

A)



B)

Kind of stress	Anxiety level	Exposure time	N. expositions	When
Open field + novel object	**	10' + 5'	2	beginning/end
Forced swimming	****	10'	2	first 2 weeks
Water Maze	***	15'	2	beginning/end
Startle response	***	15'	3	once a week
Bright light (300 lux)	****	30'	3	once a week

Elevated platform	****	120’	4	once a week
Elevated Plus Maze	**	5’	2	7 days before the beginning/end
Predator odour	***	60’	3	first 2 weeks
Fear cond. 0.4 mA training	***	5.5’	1	end
Fear cond. 0.4 mA test	***	8’	1	end

C)

Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Open field+ Novel object	Water maze	Startle response	Bright light	Elevated platform	Predator odour	Forced swim
Day 8	**Day 9**	**Day 10**	**Day 11**	**Day 12**	**Day 13**	**Day 14**
Elevated platform	Startle response	Predator odour	Bright light	Elevated platform	Forced swim	Predator odour
Day 15	**Day 16**	**Day 17**	**Day 18**	**Day 19**	**Day 20**	**Day 21**
Bright light	Startle response	Water maze	Open field	EPM+ Elevated platform	Fear conditioning training	Fear conditioning test

Fig. 5. *A) Experimental design of the study. Seven days before the beginning of the CMS procedure, the animals were subjected to an EPM, to classify them in “high anxiety” or “low anxiety”, depending on the anxiety levels demonstrated in the behavioural test. Afterwards, they were exposed to the stress procedure for 21 days, as indicated by the red arrow, and at the end of the stress procedure they were immediately exposed to another EPM, to verify their final anxiety level; finally, after 24 hours they were sacrificed. B) The scheme shows the different kinds of stress to which the animals were subjected during the CMS procedure; the anxiety level induced in the animals (represented by the number of asterisks); the duration of each stress (in minutes); the number of expositions to each stress during the 21 days and in which phase of the study each stress was applied. C) The table shows the kind of stress/stressors to which the animals were exposed every single day of the treatment.*

3.2.1.3 Chronic social defeat stress in rats and tree shrews

The social conflict paradigm was performed at the Clinical Neurobiology Laboratory, German Primate Center, Göttingen, Germany. The behavioural experiments were conducted in accordance with the European Council Directive of 24 November 1986 (86/609/ECC) and were approved by Government of Lower Saxony, Germany.

The chronic social defeat was induced as described previously (Koolhaas *et al.,* 1997; Tornatzky and Miczek, 1994), using male Wistar rats weighting 180-200 g at the beginning of the study. They were housed in six groups of four animals per cage, with food and water *ad libitum*. The colony room was maintained at a constant temperature of 21 ± 1°C with a reversed light/dark cycle (light on: 9:00 p.m. - 9:00 a.m.). Before the beginning of the behavioural study, the animals were habituated to maintenance conditions and handled daily for two weeks. All the experimental manipulations were conducted during the dark phase of the light/dark cycle under the dim red light (< 1 Lux). Lister Hooded male rats, weighting 300-350 g at the beginning of the study, were paired with sterilized females and housed in

large plastic cages, located in a separate room from the Wistar rats, but subjected to the same maintenance conditions.

The experimental design of this study is represented in Fig. 6. The first experimental phase lasted 7 days, during which all the animals were subjected to daily handling and body weight recording. The second phase was a 7-days period, during which the Sprague-Dawley rats (intruders) of the Stress (n = 6) and the Stress + fluoxetine (n = 6) groups were daily exposed to one hour social defeat. Before the beginning of the stress procedure, the female rats were removed from the cages of the experimental male Wistar rats (residents). Thus, the Sprague-Dawley rats were removed from their home cages and introduced into the resident's cages. The intruders were attacked by the residents in typically less than a minute, after which the intruders adopted freezing and submissive postures. For the remaining hour, the intruders were enclosed in a small wire-mesh compartment within the resident's cage; thus, the intruders were protected from the direct physical contact, but they remain in olfactory, visual, and auditory contact with the residents. Afterwards, the intruders were singly housed until the end of the experiment. To avoid individual differences in the defeat intensity, each day the intruders were confronted with a different resident. Control animals were kept in a separate room for all the duration of the experimental protocol and were subjected to daily handling procedure, consisting on picking up each rat, transferring it to a novel cage with sawdust bedding for 1 hour a day, similar to the defeated animals, and then returning it to its home cage. The third experimental phase, lasting 28 days, consisted of the antidepressant treatment: the stressed rats remained in the psychosocial conflict situation and were treated daily orally with fluoxetine (5 mg/Kg) or vehicle. The animals of the Control + fluoxetine group (n = 6) received the drug daily for 28 days, while the animals of the Control group (n = 6) were injected with vehicle. During all the experimental phases, body weight was recorded daily; furthermore, to reveal changes evoked by stress and counteracted by fluoxetine treatment, a battery of behavioural tests (sucrose preference test, horizontal locomotor activity, sniffing) was performed on the same animals, according to the studies published by Rygula and collaborators (2005, 2006). On the last experimental day (day 42), all the animals were sacrificed, the brains were rapidly removed, immediately frozen by immersion in isopentane, stored at a temperature of -80°C and shipped to GlaxoSmithKline laboratories for the *in vitro* experiments. A similar experimental protocol was adopted with the tree shrews, with the difference that the animals were 4/group and the administered dose of fluoxetine was 15 mg/Kg.

Coronal sections (14 µm-thick) were cut from the entire brains using a CM3050S

cryostat (Leica) approximately at 1.60 mm, -1.88 mm, -2.30 mm and -3.14 mm from Bregma (Paxinos and Watson, The Rat Brain in stereotaxic coordinates, 1998, Academic Press). The corresponding brain levels were also cut for the tree shrews brains. The slices were thaw-mounted onto polarized SuperFrost Plus slides and then stored at -80˚C until usage.

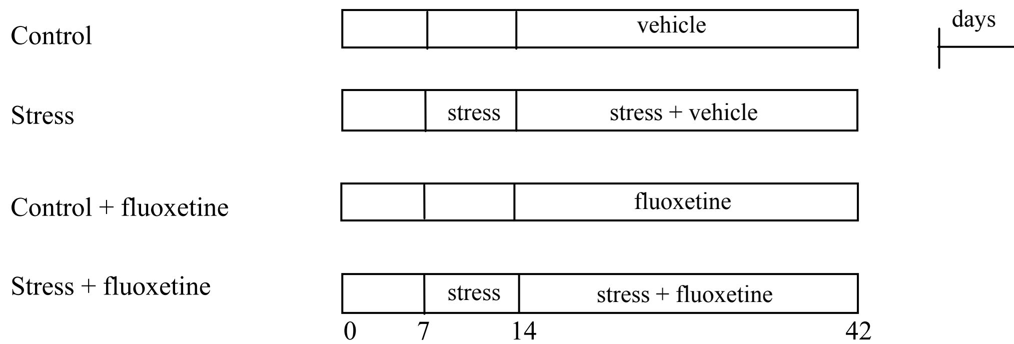


Fig. 6. *Experimental design of the study. Experimental procedures and experimental groups: Control, Stress, Control + fluoxetine, Stress + fluoxetine (n = 6 rats/group; n = 4 tree shrews/group). Phase I (days 0-7) consisted on 7 days of habituation period. During the phase II (days 7-14), the animals of the stress groups (Stress and Stress + fluoxetine) were submitted to daily psychosocial conflict, whereas the animals of the control groups (Control and Control + fluoxetine) were handled daily. During the phase III (days 14-42), the animals remained in the psychosocial conflict situation and received fluoxetine (Stress + fluoxetine: 5 mg/Kg/day for rats or 15 mg/Kg/day for tree shrews) or vehicle (Stress). Control animals remained undisturbed and received vehicle (Control) or similar drug treatment (Control + fluoxetine). On the last experimental day (day 42), the animals were sacrificed.*

3.2.2 In vitro experiments

3.2.2.1 Riboprobes preparation

The NPY rat riboprobe was made from a 508 bp cDNA of the entire NPY sequence, subcloned in a pGEM4Z plasmid vector (NM_012614; courtesy of Dr. Joseph Rimland, GlaxoSmithKline, Verona, Italy). The rat Y_1 riboprobe corresponded to a 245 bp cDNA fragment of the Y_1 receptor (genebank accession number X95507), spanning over the 4th and the 5th transmembrane regions. This cDNA was subcloned into a Bluescript II SK vector (generously provided by Dr. Ingrid Lundell, Uppsala University, Sweden). The rat Y_2 cDNA was generated based on the patent sequence (WO 95/21245) and it was a 423 bp fragment, spanning from the amino acids 651-1073 of the receptor, and subcloned in a pBSKSII vector (courtesy of Dr. Joseph Rimland). The rat Y_5 riboprobe was made from a 600 bp cDNA of the entire Y_5 sequence (NM_012869), spanning from the amino acids 703-1302 of the receptor and then subcloned in a pCRII-TOPO vector. The probes specificities were

evaluated with the public domain program Basic Local Alignment Search Tool (BLAST) in the NCBI package (http://www.ncbi.nlm.nih.gov) and each sequence did not display any significant similarity with other sequences in the Non-Redundant database at NCBI.

Once subcloned in plasmid vectors, prior to the transcription, the plasmids were linearized with appropriate restriction enzymes to generate the antisense and sense riboprobes (Table 5). The RNA probes complementary to the coding sequence were transcribed from the linearized plasmid templates with 2200 Ci/mM α-[^{33}P]UTP (Amersham Biosciences) using Sp6, T7 or T3 RNA polymerase. Transcriptions occured in the presence of 100 mM dithiothreitol, 0.5 mM each of ATP, GTP, CTP and 1 μg linearized plasmid template in 5X transcription buffer for 60 minutes at 37°C. Then 1 μl DNase was added to the transcription mixture, which was subsequently incubated for 10 minutes at 37°C. The labelled probes were then separated from unincorporated nucleotides using spin columns MicroSpin™ S-200 HR (Amersham Biosciences).

	cDNA fragment	Plasmid vector	Restriction enzyme and RNA polymerase generating sense riboprobe	Restriction enzyme and RNA polymerase generating antisense riboprobe
Rat NPY	508 bp	pGEMZ4	DraI/T7	PstI/Sp6
Rat Y_1	245 bp	Bluescript II SK	PstI/T7	EcoRI/T3
Rat Y_2	423 bp	pBSKSII	BamHI/T3	XhoI/T7
Rat Y_5	600 bp	pCRII-TOPO	XhoI/Sp6	HindIII/T7

Table 5. *Principle steps used to obtain the rat riboprobes for NPY and its receptors Y_1, Y_2 and Y_5: length (in bp) of the cDNA fragments from which the riboprobes are generated; plasmid vectors in which the cDNA fragments were subcloned; restriction enzymes used to cut the cDNA fragments and RNA polymerase used to generate the sense and antisense riboprobes are reported.*

3.2.2.2 In situ hybridization

The *in situ* hybridization reaction was carried out as previously described (Hurd & Herkenham, 1993). The sections of tissue were warmed to room temperature and allowed to dry, then they were fixed in 4% formaldehyde/1X phosphate buffered saline (PBS) for 5 minutes, rinsed twice in 1X PBS and once in 0.1 M triethanolamine/0.9% sodium chloride (pH 8) and then treated with 0.25% acetic anhydride/0.1 M triethanolamine/0.9% sodium chloride for 10 minutes. The sections were then rinsed in 2X saline sodium citrate (SSC), dehydrated in graded series of ethanol (70%, 80%, 95%, 100%) and delipidated with chloroform. They were allowed to air dry before being used or were frozen at - 80°C until

use. All aqueous solutions used in the pre-hybridization phase were prepared with RNase-free water. The hybridization buffer consisted of 1 mg/ml sheared ssDNA, 500 µg/ml yeast tRNA, 2X Denhardt's solution, 20% dextran sulfate, 8X SSC and 50% formamide. Before the hybridization, the labelled probe was added to the hybridization cocktail in the concentration of $20*10^3$ c.p.m. per µl, and 150 µl of this hybridization mixture were applied to each slide. The sections of tissue were coverslipped to prevent the evaporation and the hybridization was carried out in a humified chamber overnight at 55°C. Incubation was followed by washes in graded series of SSC (2X, 1X, 0.5X, 0.1X), all at room temperature except for 0.1X SSC (53°C) and dehydration was carried out with graded ethanol solutions (50%, 70%, 90%, 95%, 100%). The slides were then air dried and exposed to Fuji Imaging plates (BAS-TR 2025) together with ^{14}C standards for 4-5 days.

3.2.2.3 Quantification

The images from the *in situ* hybridization experiments were used for semi-quantitative analysis. Light transmittance values were measured from the digitalized images using an image analysis software system (AIS 4.0, Imaging Research, St. Catharines, Ontario, Canada). Based on the known radioactivity of the ^{14}C standards relative to their transmittance levels, the light transmittance values (PLS) per mm^2 [PLS/mm^2] were converted to nCi/g using a calibration curve. The regions of interest, i.e. cingulate cortex, septum, CA regions and dentate gyrus of hippocampus, amygdaloid and hypothalamic nuclei were chosen for their known role in depression and stress-related disorders and were defined by anatomical landmarks in conjunction with a rat brain atlas (Paxinos and Watson, The Rat Brain in stereotaxic coordinates, 1998, Academic Press). Generally, for each subject, two consecutive sections were considered, based on the anatomy, and the regions were analyzed bilaterally. The measurements of each specific brain region were taken by individually tracing the structures on the TV monitor with a cursor.

3.2.2.4 Statistical analysis

Statistical evaluations of the differences among the experimental groups were assessed using the "Statistica 6.0" software package for the analysis of variance (ANOVA). For each region, the effects of the strain (FSL/FRL in the Flinders rats; HA/LA in the rats exposed to chronic mild stress) or of the pharmacological treatment (fluoxetine/vehicle in the chronic social stressed animals) and of the stress exposure (stress/no stress) on the dependent variable (NPY and its receptors mRNA expression) were analyzed by a two-way ANOVA, followed by a

Tukey-Kramer post-comparison test. All the results were expressed as mean ± S.E.M. and in all the statistical analysis the p-value was considered significant if lower than 0.05.

3.3 NPY Y_2 RECEPTOR mRNA EXPRESSION IN AMYGDALA AND TEMPORAL CORTEX OF PSYCHIATRIC SUBJECTS

3.3.1 Tissues collections

Coronal sections of human amygdala (14 µm-thick, frozen) were obtained from the Stanley Foundation Neuropathology Consortium which collected the brains under approved ethical guidelines. Four groups were studied: major depression, bipolar disorder, schizophrenia and normal control (15 subjects per group). The demographic information is presented in Table 6. The psychiatric diagnosis was established independently by two senior psychiatrists, using DSM-IV criteria based on the information obtained from hospital records, pathologists, and/or interviews with family members or treating professionals (Torrey *et al.,* 2000). The groups had been matched for age, gender, post-mortem interval (PMI, time between the death of the individuals and the moment in which the brain tissues were frozen or fixed) and brain hemisphere. The brains studied had also been matched for mRNA stability (GAPDH and actin) and for pH. All the demographic information and the documented medical data (e.g. lifetime fluphenazine antipsychotic treatment) about the subjects were provided by the Stanley Foundation Neuropathology Consortium. Information was also provided as to the substances of abuse history (marijuana, cocaine, nicotine and alcohol), specifying if the subjects were "current drug users", based on a documented history of drug use, abuse or dependence diagnosis, "past users", defined as prior but not present drug use, or "no users", defined as no current or past drug use history.

	Control	**Major Depression**	**Bipolar Disorder**	**Schizophrenia**
Age in years	48.1	46.5	42.3	43.6
(range)	29-68	30-65	25-61	25-62
Gender				
male	9	9	9	9
female	6	6	6	5
Ethnic origin				
Caucasian	14	15	14	12
Afro-Carribean	1	-	1	-
Asian	-	-	-	1
PMI, hours	23.7	27.5	32.5	34.2
(range)	8-42	7-47	13-62	12-61
pH	6.3	6.2	6.1	6.1
(range)	5.8-6.6	5.6-6.5	5.8-6.5	5.8-6.6

Emisphere side				
Right	7	6	8	6
Left	8	9	9	9
Cause of death				
Suicide	0	7	9	4
Cardiopulmonary	13	7	4	8
Accident	2	0	1	2
Other	0	1	1	1
Antidepressant	0	9	7	5
Lithium	0	1	4	2
Antipsychotic (fluphenazine)	0	0	12	14

Table 6. *Demographic information obtained from the Stanley Foundation Neuropathology Consortium on the brain specimens examined.*

3.3.2 Probe preparation

The human Y_2 receptor riboprobe was generated from a 629 bp fragment of the human Y_2 cDNA (Rimland *et al.,* 1996), spanning over the coding region of the receptor, from the second to the sixth transmembrane domains. This fragment was successively subcloned in a PBKSII vector and used to generate RNA probes. Before the transcription, the plasmid was linearized with the ApaI and EcoRI restriction enzymes, generating respectively sense and antisense riboprobes. RNA probes complementary to the coding sequences were then transcribed from the linearized plasmid template with 2200 Ci/mM α-[^{33}P]UTP (Amersham Biosciences) using T7 and T3 RNA polymerases. The transcription reactions and the *in situ* hybridization were conducted as previously described (section 3.2.2.1).

3.3.3 Quantification

The slides were exposed to Fuji Imaging plates (BAS-TR 2025) together with ^{14}C standards for 4-5 days. Light transmittance values were measured from the digitalized images using an image analysis software system (AIS 4.0, Imaging Research, St. Catharines, Ontario, Canada). Based on the known radioactivity of the ^{14}C standards relative to their transmittance levels, the light transmittance values (PLS) per mm^2 [PLS/mm^2] were converted to nCi/g using a calibration curve. The regions of interest, such as various nuclei of the amygdala (lateral nucleus, basal intermediate division, basal magnocellular division, accessory basal nucleus) and of the cerebral cortex (entorhinal cortex, superficial and deep layers of the temporal cortex) were defined by anatomical landmarks. Generally, for each subject, two consecutive sections were considered and a minimum of six measurements for each region were taken and averaged. Background signal in the adjacent white matter was subtracted

from the averaged values. The measurements of each specific brain region were taken by individually tracing the structures on the TV monitor with a cursor.

3.3.4 Statistical analysis

The analysis of covariance (ANCOVA) using "Statistica 6" software package was performed to determine the possible differences among the various pathological groups on the Y_2 receptor mRNA expression levels in the brain regions analyzed. The independent variables (age, PMI, gender, hemisphere side and documented history of stimulant substances) were included in the statistical model if results from ANCOVA analysis were significant for that specific variable ($p < 0.05$). The significant differences obtained from the ANCOVA analysis were further assessed by Tukey–Kramer post-hoc comparison. The influence of suicide as a cause of death, the age of disease onset and the duration of the disease on the mRNA expression levels was determined only in the psychiatric groups. All the data were expressed as mean ± S.E.M.

4. RESULTS

4.1 CHARACTERIZATION OF THE NPY Y_2 KO MICE

4.1.1 In vivo experiments

4.1.1.1 LABORAS system

The behaviours of the NPY Y_2 KO and WT male mice examined in basal conditions with the LABORAS system were separately evaluated in the dark phase (6 p.m. - 6 a.m.) and in the light phase (6 a.m. - 6 p.m.) of the day. The statistical analysis performed with the Student's t-test did not report significant differences between the Y_2 KO and WT mice in the locomotion activity, grooming, feeding and drinking behaviours, while a significant increase of the climbing activity ($p < 0.05$) and of the total distance ($p < 0.01$) was evidenced in the Y_2 KO mice compared to the WT in the dark phase (Fig. 7). Moreover, in all the examined behaviours, both in the Y_2 KO and in the WT mice, a significant reduction of all the activities was observed during the light phase compared to the dark phase.

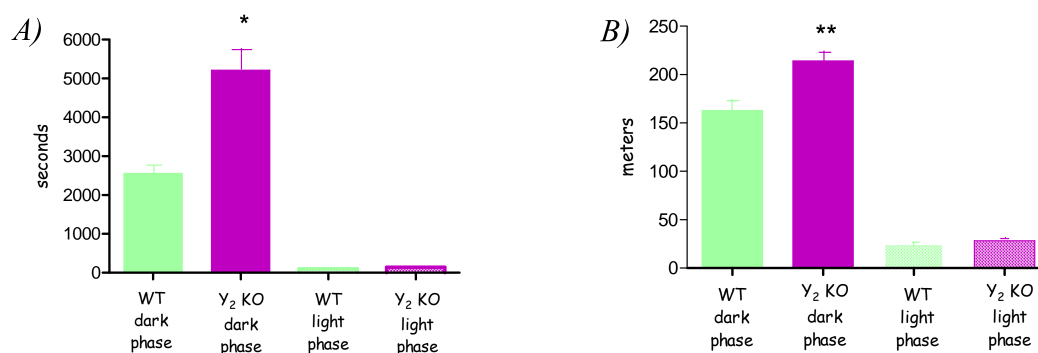


Fig. 7. *Graphical expression of the two basal behavioural parameters, climbing (A) and total distance (B), evaluated with the LABORAS system (mean ± S.E.M.), in the WT and Y_2 KO mice during the dark and light experimental phases.*
*A) time, expressed in seconds, spent in the climbing activity: comparison between WT and Y_2 KO male mice (n = 15-16) in the dark and light phase of the day (WT, dark phase: 2559.78 ± 381.9; Y_2 KO, dark phase: 5214.99 ± 729.52; WT, light phase: 104.02 ± 34.37; Y_2 KO, light phase: 141.45 ± 23.39); * $p < 0.05$.*
*B) total distance, expressed in meters: comparison between WT and Y_2 KO male mice (n = 15-16) in the dark and light experimental phases (WT, dark phase: 162.83 ± 12.41; Y_2 KO, dark phase: 213.96 ± 11.34; WT, light phase: 25.76 ± 2.17; Y_2 KO, light phase: 28.20 ± 1.62); ** $p < 0.01$.*

The Student's t-test did not evidence significant differences between the Y_2 KO and the WT female mice on the same basal behavioural activities analyzed for the male mice during both

the dark and light phases. However, as observed for the male, all the behaviours were significantly reduced during the light phase compared to the dark phase.

4.1.1.2 Elevated plus maze (EPM)

To identify eventual differences in the anxiety-related behaviour, the mice were exposed to 5 minutes of EPM, but the Student's t-test did not reveal significant differences in terms of time spent by the WT and Y_2 KO male mice in the open and closed arms of the plus maze. No differences between the two groups of mice were also observed in the same experiment preceded by 5 minutes of restraint stress. However, both WT and Y_2 KO mice displayed a decreased number of entries in the open arms of the plus maze after the stress exposure, but the statistical significance was not reached (Fig. 8).

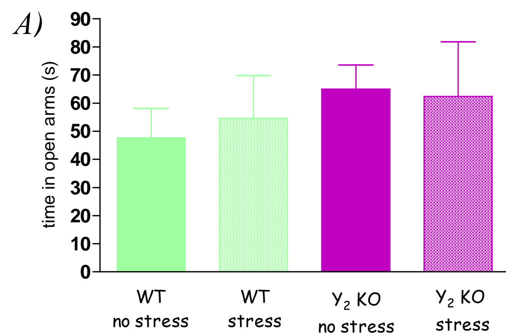
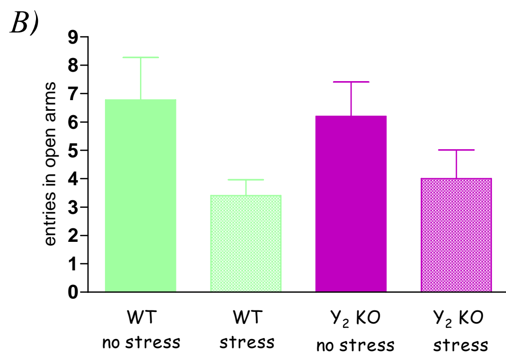
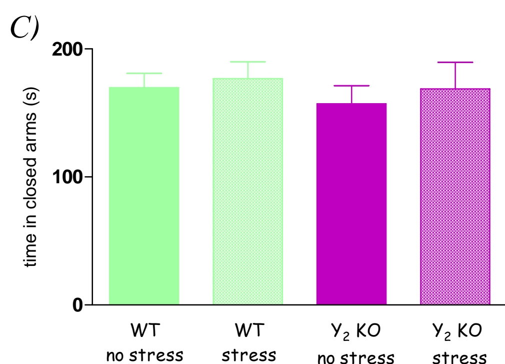


Fig. 8. *Elevated plus maze (EPM). The data are expressed as mean ± S.E.M. Comparison between WT and Y_2 KO male mice (n = 9-10), not exposed or previously exposed to 5 minutes of restraint stress.*
A) Time, expressed in seconds, spent in the open arms of the plus maze by the four groups of animals (WT, no stress: 47.67 ± 10.52; WT, stress: 58 ± 16.57; Y_2 KO, no stress: 65.75 ± 8.57; Y_2 KO, stress: 65 ± 21.59).



B) Number of total entries in the open arms of the plus maze in the four groups of mice (WT, no stress: 6.78 ± 1.49; WT, stress: 3.56 ± 0.6; Y_2 KO, no stress: 6.20 ± 1.21; Y_2 KO, stress: 4.13 ± 1.14).



C) Time, expressed in seconds, spent in the closed arms of the plus maze by the four groups of animals (WT, no stress: 169.67 ± 11.28; WT, stress: 176.44 ± 14.77; Y_2 KO, no stress: 157.1 ± 14.14; Y_2 KO, stress: 165.88 ± 23.44).

4.1.1.3 Forced Swim Test (FST)

This test evaluates the ability of the animals to cope with stressful events. Their goal consists on reaching safety through swimming although no escape route exists. Immobility or floating in this test is thought to reflect a situation in which the animals give up any attempt to escape from the water. The statistical analysis did no show behavioural differences, expressed as time of immobility, between the WT and Y_2 KO male and female mice, both in the 6 minutes and in the 10 minutes experiments (Fig. 9).

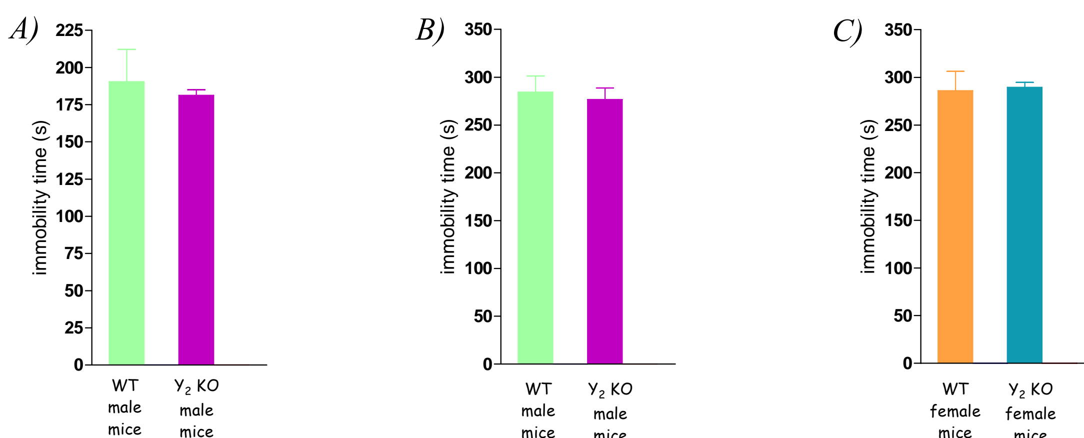


Fig. 9. *Forced swim test (FST). The data are expressed as seconds of immobility (mean ± S.E.M.).*
*A) FST - 6 minutes: comparison between WT and Y_2 KO male mice (n = 8 mice/group) during the last 4 minutes of test (WT mice: 190.38 ± 7.71; Y_2 KO mice: 183.5 ± 3.30; * $p < 0.05$).*
*B) FST - 10 minutes: comparison between WT and Y_2 KO male mice (n = 8 mice/group) during the last 5 minutes of test (WT mice: 284.38 ± 6.02; Y_2 KO mice: 276.63 ± 12.18; * $p < 0.05$).*
*C) FST - 10 minutes: comparison between WT and Y_2 KO female mice (n = 8 mice/group) during the last 5 minutes of test (WT mice: 286 ± 7.16; Y_2 KO mice: 289.5 ± 5.2; * $p < 0.05$).*

4.1.1.4 FST after desipramine treatment

The statistical analysis (one-way ANOVA followed by Duncan's test) of the immobility time in the 10 minutes FST evidenced that a significant reduction of the immobility time between 2-6 minutes in the WT male mice compared to the WT mice treated with vehicle was induced by the highest administered dose of desipramine (20 mg/Kg, i.p.; $p < 0.05$; Fig. 10). Other significant differences were not reported in the three groups of treatment of Y_2 KO mice or between WT and KO mice belonging to the same group of treatment.

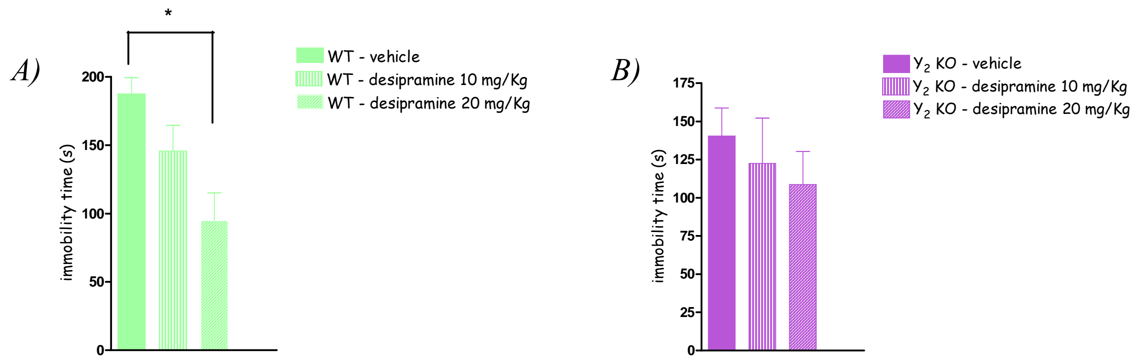


Fig. 10. *Forced swim test (FST), 10 minutes. The data, expressed as seconds of immobility, were calculated between 2-6 minutes of test (mean ± S.E.M). The test was performed on: (A) WT male mice (n = 8) and (B) Y_2 KO male mice (n = 8). 30 minutes before the beginning of the FST, they were injected i.p. with vehicle, 10 mg/Kg or 20 mg/Kg desipramine; (WT, vehicle:187.4 ± 12; WT, 10 mg/Kg:145.6 ± 19; WT, 20 mg/Kg: 94.9 ± 20.2; Y_2 KO, vehicle: 140.4 ± 18.3; Y_2 KO, 10 mg/Kg: 122.5 ± 29.6; Y_2 KO, 20 mg/Kg: 108.6 ± 21.7); * $p < 0.05$.*

In the same experiment performed on female mice, significant differences in the immobility time between 2-6 minutes of test were observed among the different groups of treatment both in the WT and in the Y_2 KO mice. The one-way ANOVA analysis followed by the Duncan's test showed a reduction of the immobility time both in the WT and Y_2 KO mice injected i.p. with 10 mg/Kg desipramine, compared to the animals of the same group injected with vehicle ($p < 0.05$ in the WT group and $p < 0.01$ in the Y_2 KO group; Fig. 11).

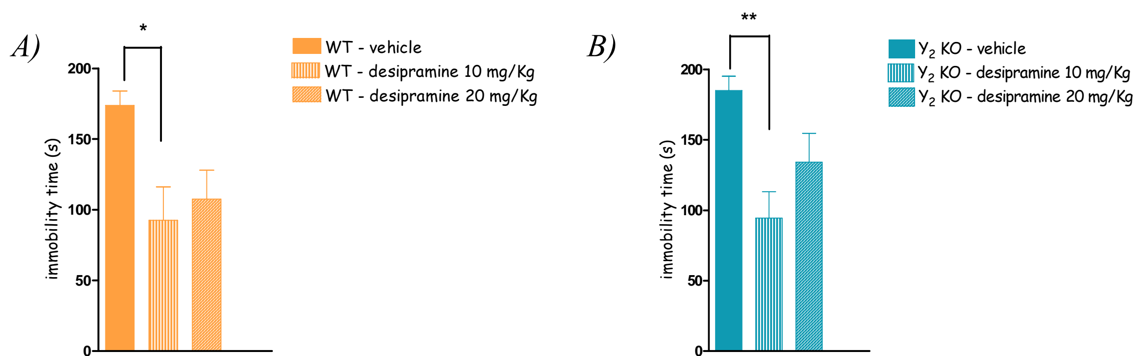


Fig. 11. *Forced swim test (FST), 10 minutes. The data, expressed as seconds of immobility, were calculated between 2-6 minutes of test (mean ± S.E.M). The test was performed on: (A) WT female mice (n = 10 - 11) and (B) Y_2 KO female mice (n = 10 - 11). 30 minutes before the beginning of the FST, they were injected i.p. with vehicle, 10 mg/Kg or 20 mg/Kg desipramine; (WT, vehicle:173.8 ± 10.1; WT, 10 mg/Kg: 92.5 ± 23.6; WT, 20 mg/Kg: 107.6 ± 20.5; Y_2 KO, vehicle: 185 ± 10.2; Y_2 KO, 10 mg/Kg: 94.5 ± 18.7; Y_2 KO, 20 mg/Kg: 134.2 ± 20.3); * $p < 0.05$; ** $p < 0.01$.*

4.1.1.5 ACTH and corticosterone levels after FST at 21°C

A two-way ANOVA statistical analysis (group of mice and stress) demonstrated that 5 minutes of FST at 21°C induced a stressful effect on the animals, both the WT and the Y_2 KO mice, which reported a significant increase ($p < 0.001$) of the ACTH and corticosterone levels (Fig. 12). Other significant differences between the two groups of animals were not detected: WT and Y_2 KO mice reported similar levels of the two analyzed hormones. In addition, no significant effects due to the interaction between the two groups of mice and the stress exposure were detected.

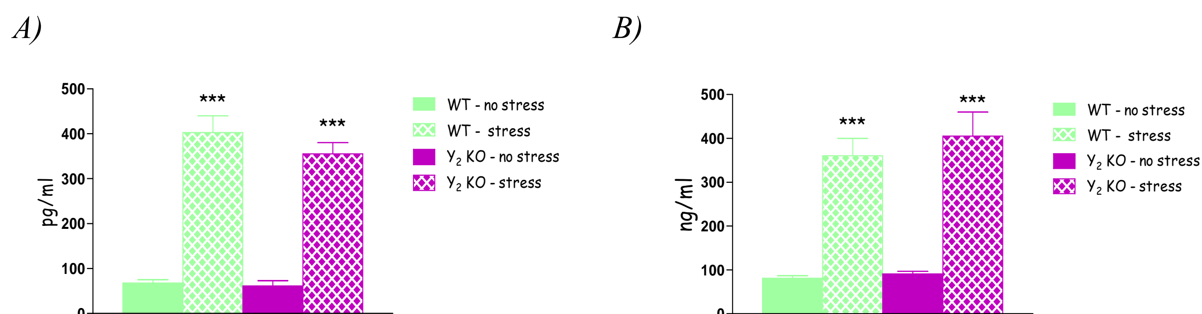


Fig. 12. *A) Plasma ACTH levels, given as pg/ml (mean ± S.E.M.), measured from blood samples of WT and Y_2 KO male mice (n = 8), not stressed or stressed with 5 minutes FST at 21ºC; *** $p < 0.001$.*
*B) Serum corticosterone levels, given as ng/ml (mean ± S.E.M.), measured from blood samples of WT and Y_2 KO male mice (n = 8), not stressed or stressed with 5 minutes FST at 21ºC; *** $p < 0.001$.*

4.1.2 In vitro experiments

4.1.2.1 Real Time-PCR reaction

The agarose gel electroforesis result was visualized by using the VERSA DOC Imaging System (BIO-RAD) and the software Quantity One (v. 4.5.1), which evidenced that the RT-PCR products, corresponding to the Y_2 receptor sequences delimited by the previously reported couples of primers (section 3.1.2.1), were only obtained from the reactions in which the hippocampal cDNAs of the WT mice had been used as substrates and in which the Super Script II enzyme had been added to the reaction (RT+) (two 303 bp bands and one 489 bp band in the higher section of the agarose gel and one 801 bp band in the lower section). The reactions in which the Y_2 KO mice cDNAs were included but the Super Script II enzyme was not added (RT-) gave no bands, as expected (Fig. 13).

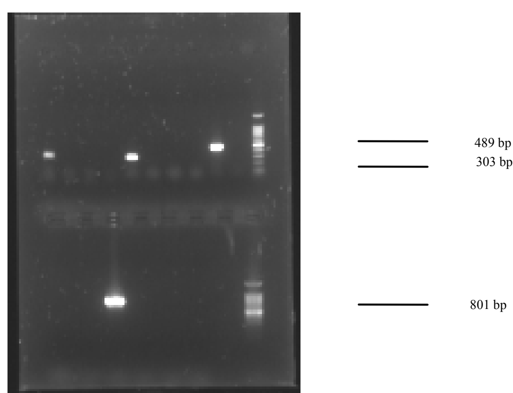


Fig. 13. *Agarose gel electrophoresis result.*

4.1.2.2 Receptor autoradiography

To demonstrate the presence of the Y_2 receptors in the WT mice and their absence in the Y_2 KO, some coronal mouse brain sections, cut approximately at -1.34 mm from Bregma (Paxinos and Watson, The Mouse Brain in stereotaxic coordinates, 1997, Academic Press) were incubated with 25 pM [^{125}I]PYY (Amersham), which binds indistinctively all the NPY receptors (Gobbi *et al.,* 1999). High levels of specific [^{125}I]PYY binding were observed in the hippocampal area and moderate levels were found in the amygdala and hypothalamic regions of the WT mice (T.B., Fig. 14A), while the Y_2 KO mice did not display [^{125}I]PYY binding, especially in the hippocampus (T.B., Fig. 14C). However, the addition of 1μM non radioactive NPY to [^{125}I]PYY (non specific binding, N.S.B.) displaced the specific binding both in the WT and in the Y_2 KO mice (Fig. 14B,D).

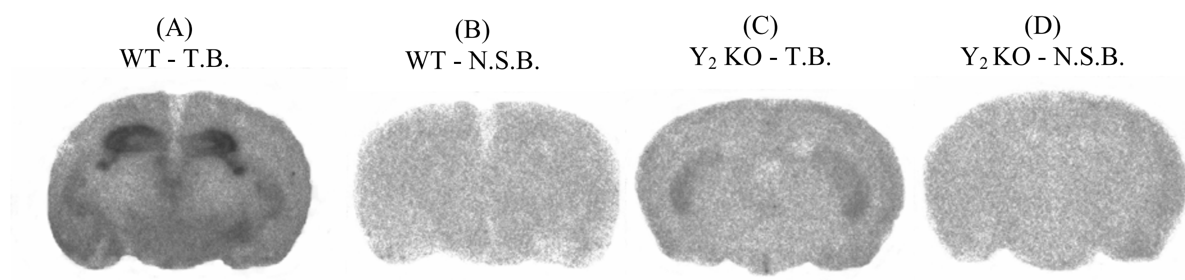


Fig. 14. *Receptor autoradiography for Y_2 receptors. Representative images of mouse coronal brain sections, approximately at -1.34 mm from Bregma. Effect of the total binding (T.B.) of the ligand [^{125}I]PYY in the WT (A) and Y_2 KO (C) mice and effect of the non specific binding (N.S.B.), obtained adding 1 μM of non radioactive NPY to [^{125}I]PYY, in the WT (B) and Y_2 KO (D) mice. In the N.S.B. (B,D) the signal corresponding to the Y_2 receptors is absent, while a specific labeling is present in the WT mice T.B. (A), but not in the Y_2 KO T.B.(C). Scale bar = 0.25 mm.*

4.1.2.3 In situ hybridization

The *in situ* hybridization reactions performed on coronal mouse brain sections, approximately at -1.34 mm from Bregma (Paxinos and Watson, The Mouse Brain in stereotaxic coordinates, 1997, Academic Press), with a rat Y_2 receptor riboprobe demonstrated a difference between the WT (A) and Y_2 KO mice (B) in the Y_2 mRNA distribution. A high signal, especially in the hippocampus, hypothalamus and amygdala, was observed in the WT mice, while the Y_2 KO mice did not display signal in the hypothalamic area and in the amygdala, whereas it was found in the hippocampus, even if with a lower intensity than in the WT mice.

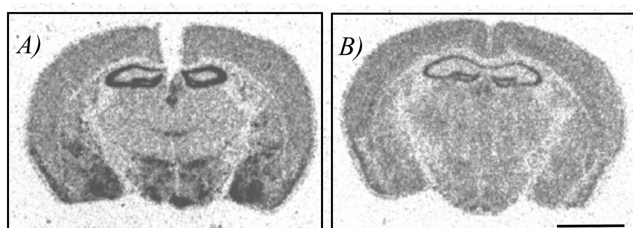


Fig.15. *Representative images of the Y_2 receptor mRNA distribution in coronal brain sections of a WT mouse (A) and a Y_2 KO mouse (B), approximately at -1.34 mm from Bregma. Scale bar = 0.25 mm.*

4.1.2.4 Real-Time quantitative PCR reaction

A possible difference between C57BL/6 and 129SvJ mouse strains in terms of basal NPY mRNA levels was analyzed using the RT-qPCR technique in the hypothalamus, a brain region in which the highest NPY levels have been observed (Morris, 1989).

The analysis of the mouse GADPH mRNA expression as an internal reference was included to normalize the data for RNA quantity and quality. The gene expression data were statistically evaluated by analysis of covariance (ANCOVA), considering the expression of GAPDH as a covariate, under the hypothesis that it was not affected by changes in the two different mouse strains. The covariance efficiency factor was determined to be close to 1 (1.085), thus proving independence of the GAPDH expression from the different mouse strains. Post-hoc analysis (Dunnett's test) was performed by comparing the means of the GAPDH expression in the two mouse strains and it did not reach the statistical significance (Fig. 16).

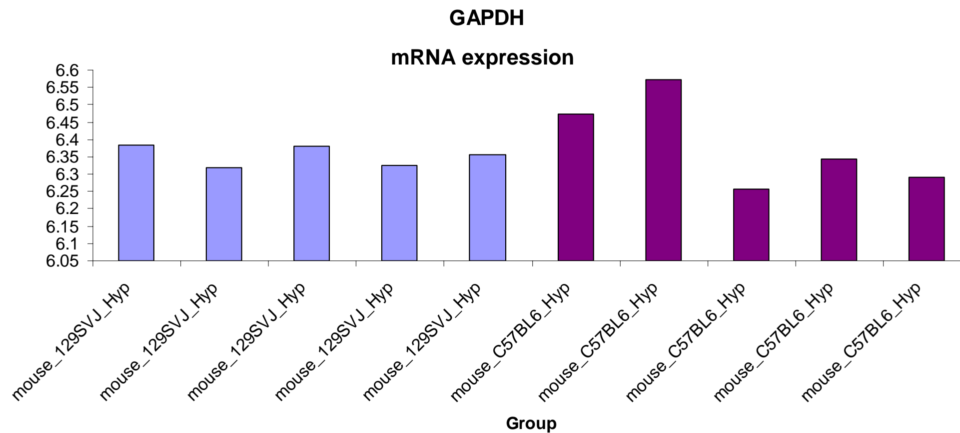


Fig. 16. *GAPDH mRNA expression levels in the hypothalamic regions of each mouse of the two strains, C57BL/6 and 129SvJ. The istograms represent an averaged value of a reaction performed in triplicate. No differences were determined in the GAPDH expression of the two mouse strains.*

The same analysis was repeated for the expression of the NPY mRNA levels in the two different mouse strains C57BL/6 and 129SvJ. The covariance efficiency factor was determined to be close to 1 (1.195), thus showing independence of the NPY expression from the different mouse strains. Post-hoc analysis (Dunnett's test) was performed by comparing the mean values of the NPY expression in the two mouse strains, but it was not statistically significant (Fig. 17).

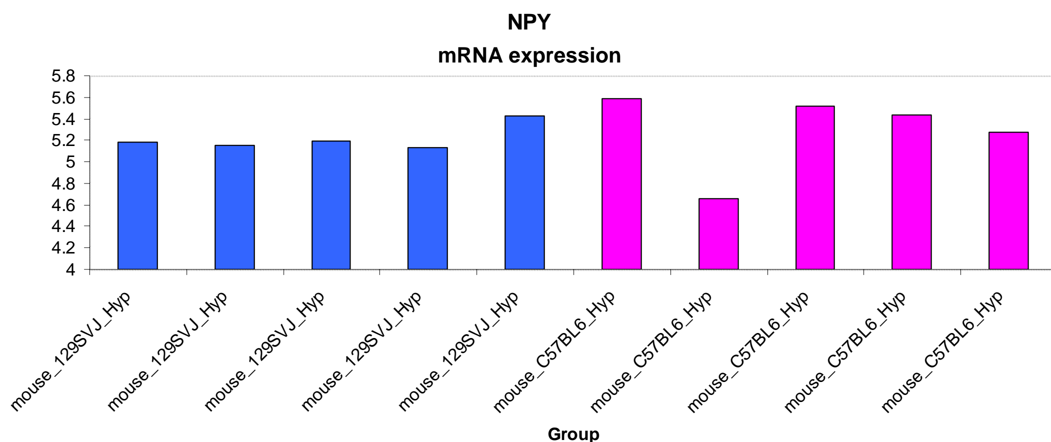


Fig. 17. *NPY mRNA expression levels in the hypothalamic regions of each mouse of the two strains, C57BL/6 and 129SvJ. The istograms represent an averaged value of a reaction performed in triplicate. No differences were determined in the NPY expression of the two mouse strains.*

4.2 ANIMAL MODELS OF DEPRESSION

4.2.1 Flinders Sensitive Line rats

4.2.1.1 NPY mRNA expression

The pattern of the NPY mRNA expression in the FSL rats was consistent with previous description of the NPY mRNA distribution, with scattered hybridization signals found mainly in the cerebral cortex, hippocampus, striatum and hypothalamus (Morris, 1989); (Fig. 1). A significant strain difference was found in the dentate gyrus of the hippocampus, in which lower NPY mRNA levels were measured in the FSL rats compared to the FRL ($p = 0.043$; Fig. 18). No significant differences of strain or due to the stress exposure or to the interaction between strain and stress were observed in the other regions examined (cingulate cortex, CA region of the hippocampus, medial amygdala and arcuate hypothalamic nucleus).

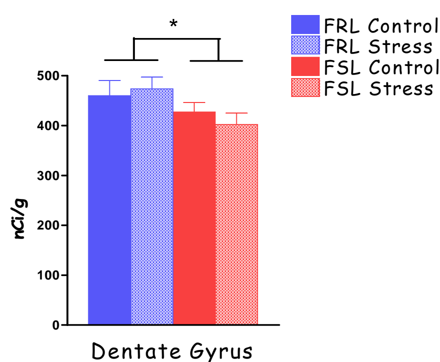


Fig. 18. *NPY mRNA expression levels in the dentate gyrus of the hippocampus (DG) in the Flinders Resistant Line (FRL) and Flinders Sensitive Line (FSL) rats, non stressed or stressed with one hour restraint. The bar graph represents the mean ± S.E.M. (n = 6 rats/group) given as nCi/g. A statistically significant difference exists between the FRL rats (n = 12 rats/group, DG = 467 ± 18.4) and the FSL rats (n = 12 rats/group, DG = 414.9 ± 14.4); * $p < 0.05$.*

4.2.1.2 Y_1 receptor mRNA expression

The expression pattern of the Y_1 mRNA distribution in the FSL rats was in line with previously reported results (Larsen *et al.,* 1993), with high levels of Y_1 hybridization signals in the neocortex, dentate gyrus of the hippocampus, several thalamic nuclei and the hypothalamic arcuate nucleus (Fig. 2). No significant differences due to the strain, the stress exposure, or to the strain x stress interaction were observed in the regions measured: cingulate cortex, septum, CA region and dentate gyrus of the hippocampus.

4.2.1.3 Y_2 receptor mRNA expression

The localization of the Y_2 mRNA expression in the FSL rats was similar to that previously reported (Gustafson *et al.,* 1997). The most intense signals were observed in the CA3 region

of the hippocampus, hypothalamic arcuate nucleus, piriform cortex, centromedial thalamic nucleus and medial amygdala (Fig. 3). No significant differences due to the strain, the stress exposure or the strain x stress interaction were found in the Y_2 mRNA levels in the analyzed regions: CA3, medial amygdala and arcuate nucleus of the hypothalamus.

4.2.1.4 Y_5 receptor mRNA expression

The expression pattern of Y_5 mRNA distribution in the FSL rats was in line with previously reported results (Gerald *et al.,* 1996), with the most intense signal in the cingulate cortex, CA3 region and dentate gyrus of hippocampus and to a less extent in a number of hypothalamic nuclei (Fig. 4). A significant interaction between strain and stress exposure was observed in the central amygdala, in which the Y_5 receptor mRNA expression levels were higher in the FSL control rats than in the FRL control rats and lower in the FSL stressed than in the FRL stressed rats ($p = 0.038$; Fig. 19). No significant differences due to the strain, the stress exposure, or to the interaction between strain and stress were observed in the other regions measured: cingulate cortex, septum, medial and basolateral amygdala, CA1, CA2, CA3 regions and dentate gyrus of hippocampus, hypothalamic arcuate, paraventricular and ventro-medial nuclei.

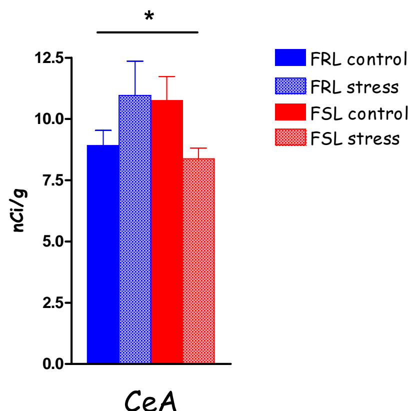


Fig. 19. *NPY mRNA expression levels in the central amygdala (CeA) in the Flinders Resistant Line (FRL) and Flinders Sensitive Line (FSL) rats, non stressed or stressed with one hour restraint. The bar graph represents the mean ± S.E.M. (n = 6 rats/group) given as nCi/g (n = 6 rats/group, CeA = 8.92 ± 0.61 in the FRL control rats; CeA = 10.96 ± 1.4 in the FRL stress rats; CeA = 10.75 ± 0.98 in the FSL control rats; CeA = 8.37 ± 0.44 in the FSL stress rats); * $p < 0.05$.*

4.2.2 Chronic mild/unpredictable stress

4.2.2.1 NPY mRNA expression

The statistical analysis of the NPY mRNA expression levels evaluated in the four experimental groups of rats (low anxiety – no stress, low anxiety – stress, high anxiety – no stress, high anxiety – stress) showed a basal difference between the two groups of rats defined low anxiety (LA) and high anxiety (HA) in the medial amygdala, with higher NPY

mRNA levels in the HA rats compared to the LA (p = 0.022; Fig. 20A). In the same region, the statistical analysis did not evidence a significant effect of the CMS exposure or of the interaction between stress and anxiety levels. Moreover, in all the other regions analyzed (cingulate cortex, septum, basolateral amygdala, hippocampal CA1, CA2, CA3 regions and dentate gyrus, arcuate hypothalamic nucleus) there were no statistical significant differences. In addition, the correlation between the behavioural data (the time spent by the animals in the open arms of the plus maze) and the anatomical data (the NPY mRNA expression) was considered and a negative trend was observed in the medial amygdala (β = - 0.47; Fig. 20B), but not in the other regions.

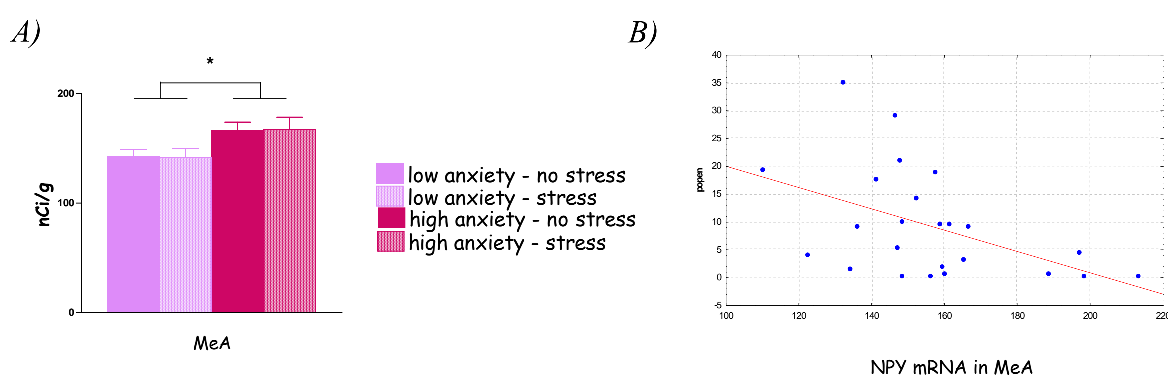


***Fig. 20**. A) NPY mRNA expression levels in the medial amygdala (MeA) of rats belonging to the low anxiety (LA) and high anxiety (HA) groups, non stressed or exposed to CMS. The bar graph represents the mean ± S.E.M. (n = 5 rats in the LA group; n = 7 rats in the HA group) given as nCi/g (LA - no stress = 142.35 ± 6.5; LA - stress = 141.5 ± 8.2; HA - no stress = 166.45 ± 7.5; HA - stress = 167.35 ± 11.1; * p < 0.05).*
B) Linear regression between the NPY mRNA levels in the MeA, given as nCi/g, in the x axis and the time, expressed in seconds, spent by the rats in the open arms of the maze in the y axis. A negative correlation is observed between the two parameters (β = -0.47).

4.2.2.2 Y_1 receptor mRNA expression

In the four experimental groups of rats, the Y_1 receptor mRNA expression was studied in the following regions: cingulate cortex, septum, central, medial and basolateral nuclei of the amygdala, hippocampal CA1, CA2, CA3 and dentate gyrus, hypothalamic paraventricular, ventro-medial and arcuate nuclei. A significant difference in the basal Y_1 receptor mRNA levels between the HA and LA rats was found in the central amygdala, with a higher expression in the HA animals (p = 0.016; Fig. 21A). However, in the dentate gyrus of the hippocampus the HA rats have lower basal Y_1 receptor mRNA levels compared to the LA (p = 0.014; Fig. 21C). No significant effect due to the CMS exposure or to the interaction

between stress and anxiety levels was observed in any analyzed regions. In addition, a negative correlation between the behavioural data (the time spent by the animals in the open arms of the plus maze) and the anatomical data (the Y_1 receptor mRNA expression) was found in the central amygdala (β = - 0.51; Fig. 21B), while in the dentate gyrus the correlation was positive (β = + 0.435; Fig. 21D). The other regions did not present any correlation.

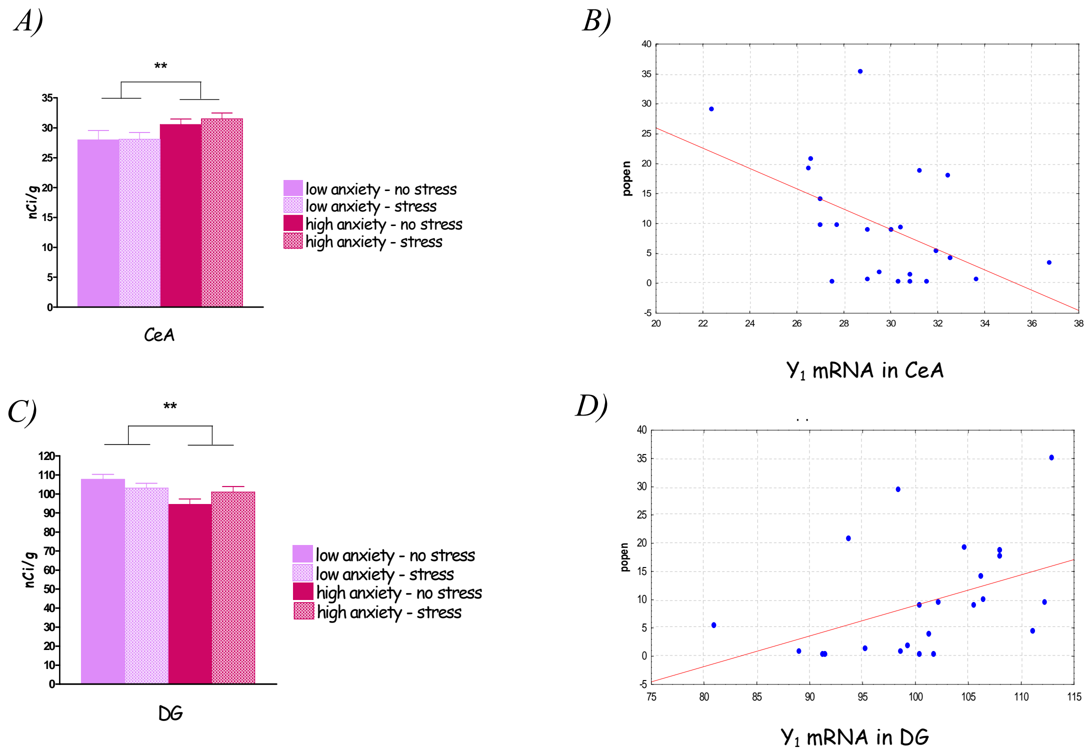


Fig. 21. *A) Y_1 receptor mRNA expression levels in the central amygdala (CeA) of rats belonging to the LA and HA groups, non stressed or exposed to CMS. The bar graph represents the mean ± S.E.M. (n = 5 rats in the LA group; n = 7 rats in the HA group) given as nCi/g (LA - no stress = 28 ± 1.6; LA - stress = 28.1 ± 1.1; HA - no stress = 30.5 ± 0.9; HA - stress = 31.5 ± 0.9; ** $p < 0.01$).*
B) Linear regression between the Y_1 receptor mRNA levels in the CeA, given as nCi/g, in the x axis, and the time, expressed in seconds, spent by the rats in the open arms of the maze in the y axis. A negative correlation is observed between the two considered parameters (β = - 0.51).
*C) Y_1 receptor mRNA expression levels in the dentate gyrus (DG) of hippocampus of rats belonging to the LA and HA groups, non stressed or exposed to CMS. The bar graph represents the mean ± S.E.M. (n = 5 rats in the LA group; n = 7 rats in the HA group) given as nCi/g (LA - no stress = 107.7 ± 2.6; LA - stress = 103 ± 2.5; HA - no stress = 94.5 ± 2.8; HA - stress = 100.1 ± 2.9; ** $p < 0.01$).*
D) Linear regression between the Y_1 receptor mRNA levels in the DG, given as nCi/g, in the x axis, and the time, expressed in seconds, spent by the rats in the open arms of the maze in the y axis. A positive correlation can be observed between the two considered parameters (β = + 0.435).

4.2.2.3 Y_2 receptor mRNA expression

The HA and LA rats did not present basal differences in the Y_2 receptor mRNA expression in

the following regions: cingulate cortex, septum, central and medial amygdala, arcuate nucleus of the hypothalamus, CA1, CA2, CA3 and dentate gyrus of the hippocampus. Moreover, no significant differences due to the CMS exposure or to the interaction between stress and anxiety levels were found.

4.2.2.4 Y_5 receptor mRNA expression

The basal Y_5 receptor mRNA expression levels were not different between HA and LA rats, however in the two groups a significant stress-induced reduction of the Y_5 receptor mRNA expression was found in the cingulate cortex ($p = 0.015$; Fig. 22A), medial amygdala ($p = 0.032$; Fig. 22B) and arcuate nucleus of hypothalamus ($p = 0.033$; Fig. 22C). However, no significant changes due to the interaction between the stress exposure and the anxiety levels were observed in the mentioned regions. All the other brain areas considered (septum, central and basolateral amygdala, paraventricular and ventro-medial hypothalamus, CA1, CA2, CA3 and dentate gyrus of hippocampus) did not show any significant changes. In addition, the existence of a correlation between the Y_5 receptor mRNA expression in all these regions and the time spent by the rats in the open arms of the maze was evaluated, but no significant results were observed.

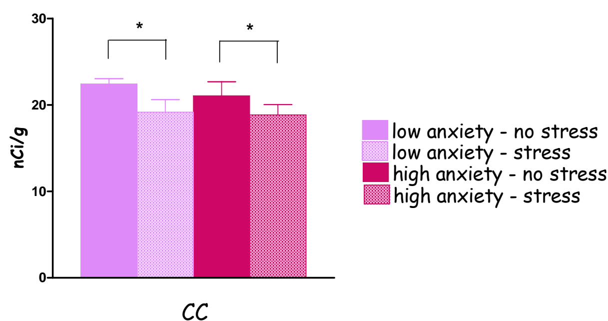
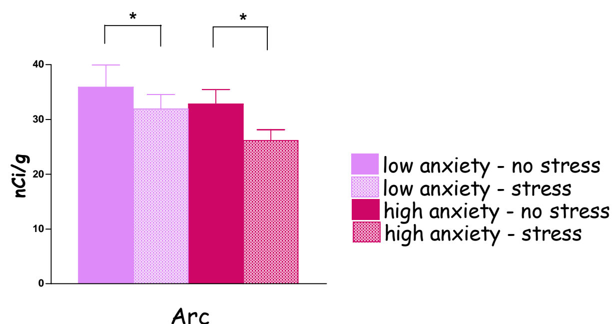
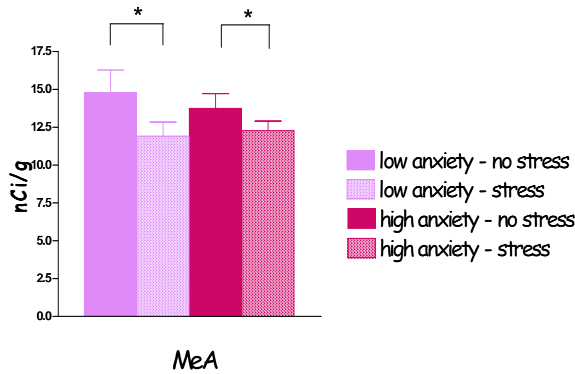


Fig. 22. A*) Y_5 receptor mRNA expression levels in the cingulate cortex (CC) of rats belonging to the LA and HA groups, non stressed or exposed to CMS. The bar graph represents the mean ± S.E.M. (n = 5 rats in the LA group; n = 7 rats in the HA group) given as nCi/g (LA - no stress = 22.4 ± 0.6; LA - stress = 19.2 ± 1.4; HA - no stress = 21 ± 1.6; HA - stress = 18.8 ± 1.2; * p < 0.05).*



*B) Y_5 receptor mRNA expression levels in the arcuate nucleus of hypothalamus (Arc) of rats belonging to the LA and HA groups, non stressed or exposed to CMS. The bar graph represents the mean ± S.E.M. (n = 5 rats in the LA group; n = 7 rats in the HA group) given as nCi/g (LA - no stress = 35.9 ± 4.1; LA - stress = 31.9 ± 2.6; HA - no stress = 32.8 ± 2.6; HA - stress = 26.2 ± 2; * p < 0.05).*



*C) Y_5 receptor mRNA expression levels in the medial amygdala (MeA) of rats belonging to the LA and HA groups, non stressed or exposed to CMS. The bar graph represents the mean ± S.E.M. (n = 5 rats in the LA group; n = 7 rats in the HA group) given as nCi/g (LA - no stress = 14.8 ± 1.5; LA - stress = 11.9 ± 0.9; HA - no stress = 13.7 ± 1; HA - stress = 12.3 ± 0.6; * $p < 0.05$).*

4.2.3 Chronic social defeat stress in rats and tree shrews

4.2.3.1 Rat NPY mRNA expression

The statistical analysis of the NPY mRNA expression levels evaluated in the four experimental groups of animals (Control, Stress, Control + fluoxetine, Stress + fluoxetine) showed a significant difference in the basal NPY mRNA expression levels in the CA3 hippocampal region (p = 0.011) of the stressed rats. In the dentate gyrus of the hippocampus, a significant effect of the interaction between chronic social stress and fluoxetine treatment was detected (p = 0.018), with a down-regulation in the stressed rats compared to the control rats and an up-regulation in the Stress + fluoxetine group compared to the Control + fluoxetine (Fig. 23). In all the other regions analyzed, such as cingulate cortex, septum, medial amygdala, CA1 and CA2 hippocampal regions and arcuate nucleus of hypothalamus, no significant changes were detected.

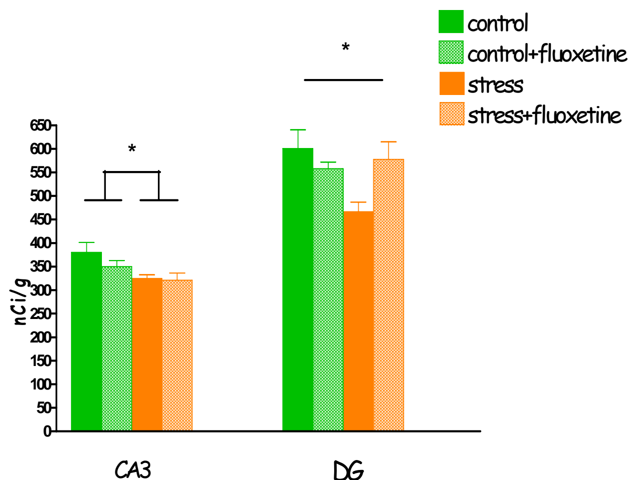


***Fig. 23.** NPY mRNA expression levels in the CA3 region and in the dentate gyrus (DG) of hippocampus of rats belonging to the Control and Stress groups, not treated or treated with fluoxetine. The bar graph represents the mean ± S.E.M. (n = 6 rats/group) given as nCi/g (CA3 = 379.915 ± 21.12 in the Control group; CA3 = 349.925 ± 12.87 in the Control + fluoxetine group; CA3 = 324.98 ± 7.45 in the Stress group; CA3 = 320.97 ± 15.49 in the Stress + fluoxetine group. DG = 600.67 ± 39.7 in the Control rats; DG = 557.93 ± 14.04 in the Control + fluoxetine rats; DG = 466.32 ± 20.36 in the Stress rats e DG = 577.47 ± 37.11 in the Stress + fluoxetine rats); * p<0.05.*

4.2.3.2 Rat Y_1 receptor mRNA expression

A significant stress x treatment interaction was found in the Y_1 receptor mRNA expression levels in the ventro-medial hypothalamic nucleus (VMH; $p = 0.036$), with a down-regulation in the stressed rats and an up-regulation in the stressed animals treated with fluoxetine (Fig. 24). In all the other regions measured (cingulate cortex, septum, central, medial and basolateral amygdala, paraventricular and arcuate hypothalamic nuclei, CA1, CA2, CA3 and dentate gyrus of hippocampus) no differences were found.

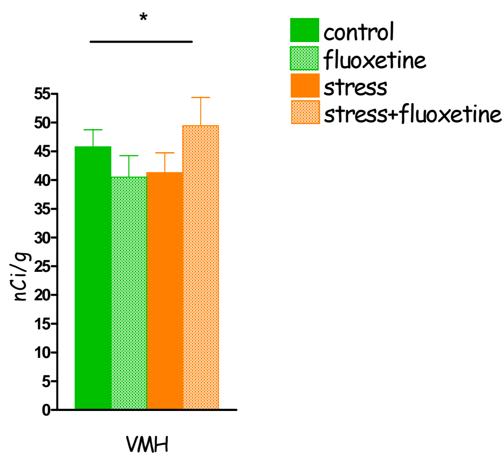


Fig. 24. *Y_1 receptor mRNA expression levels in the ventro-medial hypothalamus (VHM) of rats belonging to the Control and Stress groups, not treated or treated with fluoxetine. The bar graph represents the mean ± S.E.M. (n = 6 rats/group) given as nCi/g (VMH = 46.35 ± 2.0 in the Control rats; VMH = 40.97 ± 6.52 in the Control + fluoxetine rats; VMH = 41.98 ± 3.8 in the Stress rats and VMH = 48.31 ± 5.73 in the Stress + fluoxetine rats); * p < 0.05.*

4.2.3.3 Rat Y_2 receptor mRNA expression

A significant difference in the basal Y_2 receptor mRNA expression levels was observed in the ventro-medial hypothalamus, dorso-medial portion (VMHDM; $p = 0.027$), with lower levels found in the stressed animals (Fig. 25). There were no significant changes in the other regions considered: cingulate cortex, septum, central and medial amygdala, arcuate nucleus of hypothalamus, CA1, CA2, CA3 and dentate gyrus hippocampal regions.

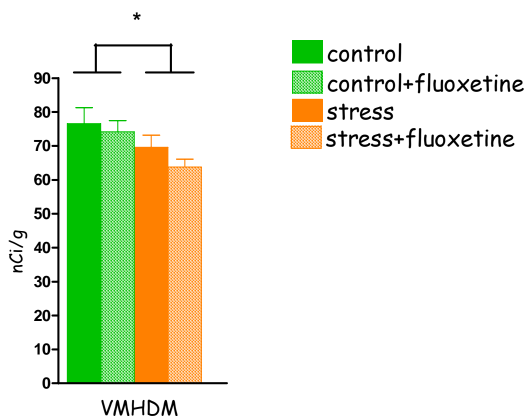


Fig. 25. *Y_2 receptor mRNA expression levels in the ventro-medial hypothalamus, dorso-medial portion (VMHDM) of rats belonging to the Control and Stress groups, not treated or treated with fluoxetine. The bar graph represents the mean ± S.E.M. (n = 6 rats/group) given as nCi/g (VMHDM = 76.57 ± 4.69 in the Control rats; VMHDM = 74.16 ± 3.3 in the Control + fluoxetine rats; VMHDM = 69.6 ± 3.58 in the Stress rats and VMHDM = 63.74 ± 2.37 in the Stress + fluoxetine rats); * p < 0.05.*

4.2.3.4 Rat Y_5 receptor mRNA expression

A significant effect due to the interaction between the stress exposure and the fluoxetine treatment was observed in the ventro-medial hypothalamus (VMH), with a down-regulation in the stressed rats compared to the control rats and an up-regulation in the stressed rats treated with the antidepressant compared to the controls treated with fluoxetine ($p = 0.041$; Fig. 26). All the other regions analyzed (cingulate cortex, septum, central, medial and basolateral amygdala, paraventricular and arcuate hypothalamic nuclei, hippocampal CA1, CA2, CA3 regions and dentate gyrus) did not reveal significant variations in the Y_5 receptor mRNA expression.

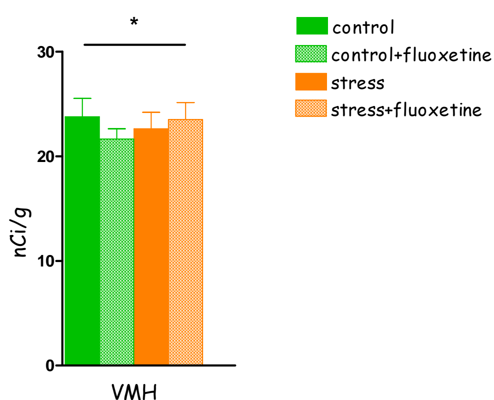


Fig. 26. *Y_5 receptor mRNA expression levels in the ventro-medial hypothalamus (VHM) of rats belonging to the Control and Stress groups, not treated or treated with fluoxetine. The bar graph represents the mean ± S.E.M. (n = 6 rats/group) given as nCi/g (VMH = 23.75 ± 1.77 in the Control rats; VMH = 21.64 ± 0.97 in the Control + fluoxetine rats; VMH = 22.62 ± 1.56 in the Stress rats and VMH = 23.5 ± 1.62 in the Stress + fluoxetine rats); *p < 0.05.*

4.2.3.5 Tree shrew NPY mRNA expression

The distribution of NPY and of its Y_1, Y_2 and Y_5 receptors mRNA in the tree shrew brain (Fig. 27) was found to be very similar to the NPY distribution in the rat brain (Figg. 1, 2, 3, 4).

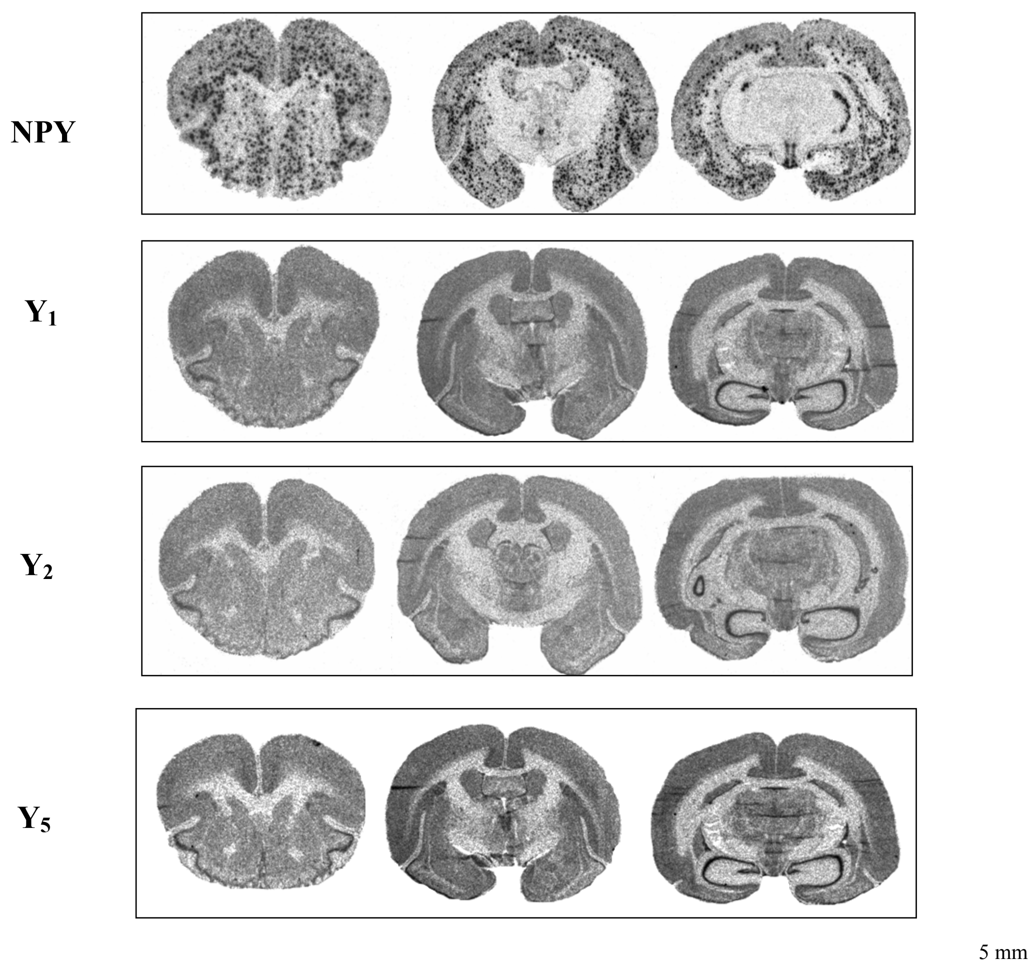


Fig. 27. *Anatomical distribution of NPY and its receptors Y_1, Y_2 and Y_5 mRNA expression in three representative levels of the tree shrew brain. Scale bar = 5 mm.*

The two-way ANOVA analysis of the NPY mRNA expression levels evaluated in the four experimental groups of animals (Control, Stress, Control + fluoxetine, Stress + fluoxetine) showed a significant increase of the NPY mRNA expression in the CA1 ($p = 0.007$) and CA2 ($p = 0.034$) hippocampal regions and in the arcuate nucleus of hypothalamus ($p = 0.01$) after exposure to stress (Fig. 28). In addition, the one-way ANOVA and the post-hoc analysis (Newman-test) were conducted, showing the presence of a *trend* (a result not reaching the statistical significance) in the CA1 hippocampal region between the Control and Stress groups of animals ($p = 0.056$), between the Control and Stress + fluoxetine groups ($p = 0.092$) and between the Control + fluoxetine and Stress groups ($p = 0.083$). The same

analysis was repeated for the CA2 region and in this case the presence of a trend was observed between the Control and Stress groups (p = 0.068), between the Control + fluoxetine and Stress groups (p = 0.051) and between Stress and Stress + fluoxetine groups (p = 0.058). In addition, in all these regions, in particular in the arcuate nucleus of hypothalamus, the effect of the chronic stress was reversed by the action of the antidepressant fluoxetine, which induced a reduction of the NPY mRNA levels, restoring the initial conditions. No significant differences were found in all the other regions analyzed, such as cingulate cortex, septum, medial amygdala, CA3 region and dentate gyrus of hippocampus.

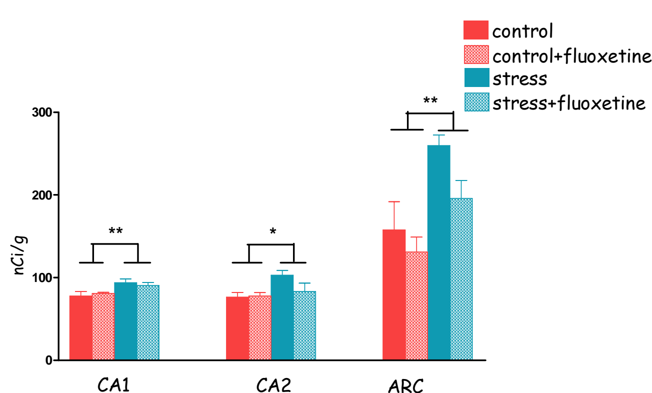


Fig. 28. *NPY mRNA expression levels in the CA1 and CA2 hippocampal regions and in the hypothalamic arcuate nucleus (ARC) of tree shrews belonging to the Control and Stress groups, not treated or treated with fluoxetine. The bar graph represents the mean ± S.E.M. (n = 4 animals/group) given as nCi/g (CA1 = 77.85 ± 5.5 in the animals of the Control group; CA1 = 80.93 ± 1.49 in the Control + fluoxetine group; CA1 = 93.99 ± 4.53 in the Stress group and CA1 = 90.57 ± 3.49 in the Stress + fluoxetine groups; CA2 = 76.61 ± 5.54 in the animals of the Control group; CA2 = 77.87 ± 4.19 in the Control + fluoxetine animals; CA2 = 102.96 ± 5.76 in the Stress group; CA2 = 83.37 ± 9.84 in the Stress + fluoxetine group; ARC = 157.83 ± 34.11 in the animals of the Control group; ARC = 131.15 ± 17.88 in the Control + fluoxetine group; ARC = 259.76 ± 12.85 in the Stress group; ARC = 195.83 ± 21.7 in the Stress + fluoxetine animals); * p < 0.05; ** p < 0.01.*

4.2.3.6 Tree shrew Y_1 receptor mRNA expression

The statistical analysis did not evidence any alteration in the tree shrew Y_1 receptor mRNA expression levels after the exposure to stress, or the treatment with fluoxetine or due to the interaction between stress and fluoxetine in all the regions considered: cingulate cortex, septum, medial and basolateral amygdala, CA1, CA2, CA3 regions and dentate gyrus of hippocampus.

4.2.3.7 Tree shrew Y_2 receptor mRNA expression

Among all the regions considered, such as cingulate cortex, septum, medial and basolateral amygdala, CA1, CA2, CA3 regions and dentate gyrus of hippocampus, the Y_2 receptor mRNA expression was found to be up-regulated only in the CA3 region after exposure to stress (p = 0.043; Fig. 29).

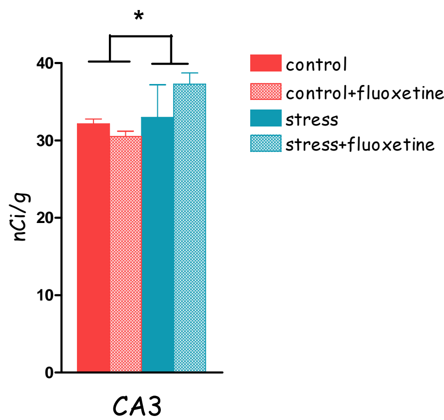


Fig. 29. *Y_2 receptor mRNA expression levels in the CA3 hippocampal region of tree shrews belonging to the Control and Stress groups, not treated or treated with fluoxetine. The bar graph represents the mean ± S.E.M. (n = 4 animals/group) given as nCi/g (CA3 = 32.15 ± 0.62 in the animals of the Control group; CA3 = 30.53 ± 0.67 in the Control + fluoxetine animals; CA3 = 32.98 ± 4.23 in the Stress group; CA3 = 37.27 ± 1.46 in the Stress + fluoxetine animals); * p < 0.05.*

4.2.3.8 Tree shrew Y_5 receptor mRNA expression

The two-way ANOVA analysis of the Y_5 receptor mRNA expression in the tree shrews reported significant changes due to the antidepressant treatment in the cingulate cortex of the stressed animals (p = 0.028) and in the same region the Y_5 mRNA levels were also affected by the interaction between the stress exposure and the fluoxetine treatment (p = 0.044; Fig. 30). In addition, the one-way ANOVA and the post-hoc analysis (Newman-test) were conducted and a significant interaction between the expression in the animals of the Stress group and the Stress + fluoxetine was demonstrated (p = 0.027), while a *trend* (p = 0.064) was found between the animals of the Control and the Stress groups. Furthermore, the Y_5 receptor mRNA expression was affected by the stress exposure in the medial amygdala (p = 0.045), while a difference due to the interaction between stress and fluoxetine was observed in the CA1 (p = 0.044) and CA2 (p = 0.0031) hippocampal regions. No significant differences were seen in all the other regions analyzed, such as septum, basolateral amygdala, CA3 and dentate gyrus hippocampal regions.

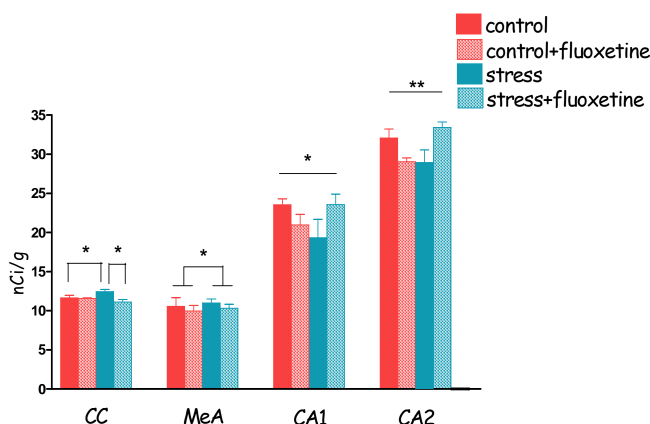
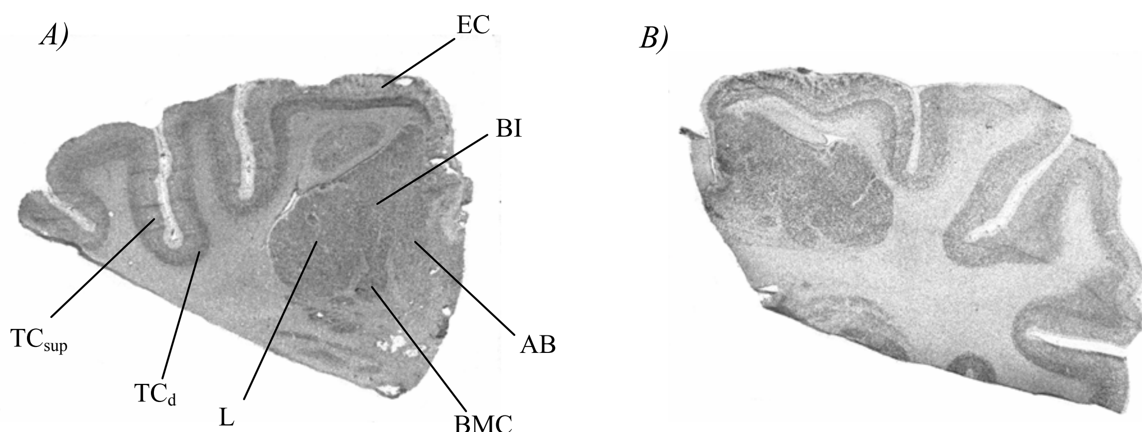


Fig. 30. *Y_5 receptor mRNA expression levels in the cingulate cortex (CC), medial amygdala (MeA), CA1 and CA2 hippocampal regions of tree shrews belonging to the Control and Stress groups, not treated or treated with fluoxetine. The bar graph represents the mean ± S.E.M. (n = 4 animals/group) given as nCi/g (CC = 11.63 ± 0.33 in the Control group; CC = 11.56 ± 0.066 in the Control + fluoxetine group; CC = 12.44 ± 0.29 in the Stress group; CC = 11.12 ± 0.31 in the Stress + fluoxetine group; MeA = 10.56 ± 1.1 in the Control tree shrews; MeA = 9.97 ± 0.71 in the Control + fluoxetine tree shrews; MeA = 10.99 ± 0.5 in the Stress animals; MeA = 10.33 ± 0.48 in the*

*Stress + fluoxetine animals; CA1 = 23.54 ± 0.76 in the Control group; CA1 = 20.96 ± 1.34 in the Control + fluoxetine animals; CA1 = 19.32 ± 2.34 in the Stress group; CA1 = 23.56 ± 1.3in the animals of the Stress + fluoxetine group; CA2 = 32.06 ± 1.13 in the Control group; CA2 = 29.03 ± 0.48 in the Control + fluoxetine animals; CA2 = 28.94 ± 1.6 in the Stress group; CA2 = 33.42 ± 0.66 in the Stress + fluoxetine animals); * $p < 0.05$; ** $p < 0.01$.*

4.3 NPY Y_2 RECEPTOR mRNA EXPRESSION IN AMYGDALA AND TEMPORAL CORTEX OF PSYCHIATRIC SUBJECTS

The Y_2 receptor mRNA distribution pattern in the human amygdala was consistent with that previously described (Caberlotto *et al.*, 1998b). Hybridization signals were observed in many nuclei of the amygdaloid complex, such as the accessory basal nucleus (AB), the basal magnocellular division (BMC), the intermediate division (BI) and the lateral nucleus (L). In addition, a high hybridization signal was observed in the adjacent entorhinal cortex (EC) and temporal cortex (TC), with a major expression in the deep layers (TC_d), compared to the superficial layers (TC_{sup}) of the temporal cortex (Fig. 31A). In all these regions, no statistical significant differences in the Y_2 receptor mRNA expression were observed in the four psychiatric groups (control, major depression, bipolar disorder and schizophrenia). However, a positive correlation between the Y_2 receptor mRNA expression and increasing age was found in all the considered regions (TC_{sup}: r = + 0.1252; TC_d: r = + 0.1865; AB: r = + 0.0176; BI + BMC: r = + 0.1236; LA: r = + 0.1194; Fig. 33A,B). On the contrary, the correlation between the Y_2 receptor mRNA expression and the post-mortem interval (PMI) was negative in all the analyzed regions (TC_{sup}: r = - 0.3234; TC_{pro}: r = - 0.3686; AB: r = - 0.2879; BI + BMC: r = - 0.2638; LA: r = - 0.3315; Fig. 33C,D). No correlation was detected between the Y_2 receptor mRNA expression and the gender of the subjects, the cerebral hemisphere analyzed, the use of antidepressant drugs, the duration of the treatment and the cause of death.



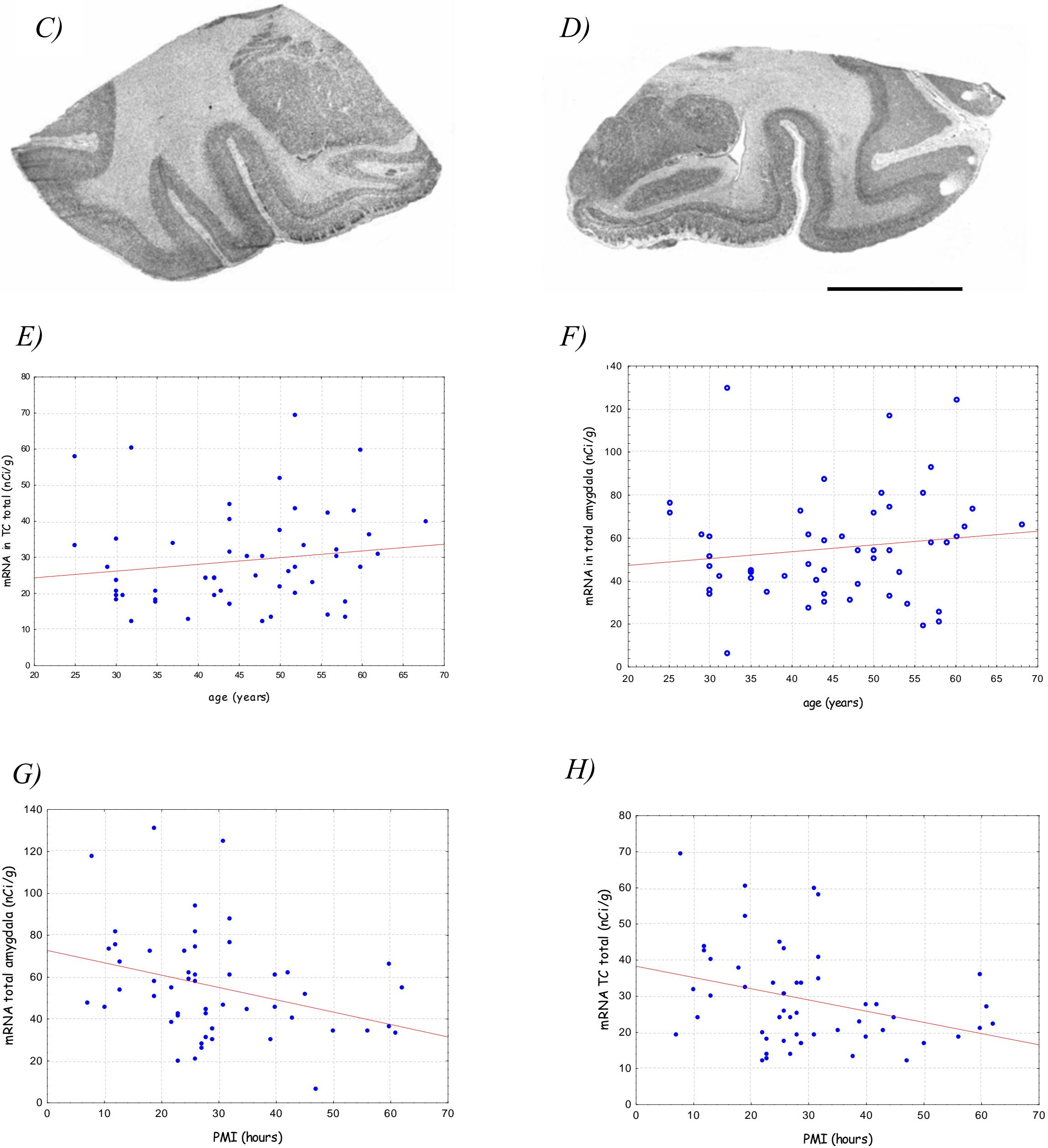


Fig. 31. *A) Representative images of the Y_2 receptor mRNA expression in coronal sections of human amygdala. L: lateral nucleus; BI: basal intermediate division; BMC: basal magnocellular division; AB: accessory basal nucleus; EC: entorhinal cortex; TC_{sup}: superficial layers of the temporal cortex; TC_d: deep layers of the temporal cortex. Scale bar = 8 mm.*

Comparison between the human Y_2 receptor mRNA expression in the temporal cortex and amygdaloid complex of a 61 years old subject (A) and of a 35 years old one (B): up-regulation of Y_2 receptor mRNA in the brain regions of the older subject. The graphical representation of the positive correlation between the Y_2 receptor mRNA expression and the age of the subjects in the temporal cortex (E) and in the amygdaloid complex (F) in also shown: the lines represent the linear regression between the Y_2 receptor mRNA levels, given as nCi/g, in the y axis, and the age of the patients, expressed in years, in the x axis.

Comparison between the human Y_2 receptor mRNA expression in the temporal cortex and amygdaloid complex in two human brain samples with 61 hours PMI (C) and 19 hours PMI (D): reduction of the Y_2 mRNA expression related to an increasing PMI in all the regions. The graphical representation of the negative correlation between the Y_2 receptor mRNA expression and the PMI in the temporal cortex (G) and in the amygdaloid complex (H) is also reported: the lines represent the linear regression between the Y_2 receptor mRNA levels, given as nCi/g, in the y axis, and the PMI of the human brain samples, expressed in hours, in the x axis.

The effect of the assumption of substances of abuse, such as marijuana, cocaine, nicotine and alcohol on the Y_2 receptor mRNA expression was also considered. A significant increase of the Y_2 receptor mRNA expression was demonstrated in the accessory basal nucleus of the amygdala of cocaine abusers, compared to subjects that had used cocaine in the past ($p = 0.0484$; Fig. 32A). Finally, lower Y_2 receptor mRNA expression levels were found in the superficial layers of the temporal cortex of cigarettes smokers subjects compared to non smokers ($p = 0.0484$; Fig. 32B). No significant effect was observed in the subjects with a history of marijuana and alcohol use.

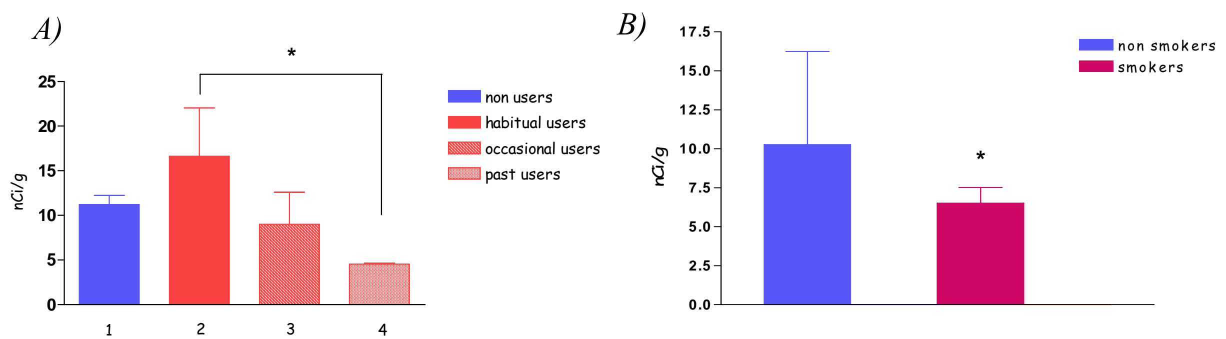


Fig. 32. *A) Human Y_2 receptor mRNA expression levels, given as nCi/g, in the accessory basal nucleus of the amygdala in relation to the cocaine assumption. A statistical significant difference is observed between the subjects defined cocaine abusers (group 2, $n = 5$, AB: 16.63 ± 5.42) and the subjects that had used this substance in the past (gruppo 4, $n = 2$, AB: 5.54 ± 0.12), who present a lower Y_2 receptor mRNA expression; * $p < 0.05$. The other two groups (group 1, non users, $n = 50$, AB: 11.2 ± 0.99 and group 3, occasional users, $n = 2$, AB: 9.02 ± 3.57) did not reach a statistical significant result.*
*B) Graphical representation of the human Y_2 receptor mRNA expression, given as nCi/g, in the superficial layers of the temporal cortex of cigarettes smokers and non smokers subjects. A significant down-regulation of the Y_2 mRNA levels is observed in the smokers group ($n = 20$, TC sup: 6.51 ± 1) compared to non smokers ($n = 10$, TC sup: 10.27 ± 1.65); * $p < 0.05$.*

5. DISCUSSION

5.1 CHARACTERIZATION OF THE NPY Y_2 KNOCKOUT MICE

The purpose of the present study consisted on improving the knowledge on the involvement of the NPY system in the pathophysiology of mood disorders, such as depression and anxiety, focusing on the role of the three major receptor subtypes in these disorders. In particular, this study on the Y_2 KO mice would investigate the hypothesis of the involvement of the Y_2 receptor subtype in anxiety and stress-related behaviours. The present research initially started with the behavioural characterization of the Y_2 KO mice, to compare it with previous data demonstrating reduced anxiety and increased stress-coping ability in these mice (Tschenett *et al.,* 2003; Redrobe *et al.,* 2003). Afterwards, the role of the Y_1 receptor would be evaluated after treating them with a Y_1 receptor specific antagonist, to assess if the lack of the Y_2 receptor would have induced the activation of a compensatory mechanism involving the Y_1 receptor subtype, thus supporting a role for both the receptors in the anxiety- and depressive-related behaviours.

A general basal behavioural analysis was initially performed to compare the behavioural activities of the Y_2 KO male mice and of their WT in baseline conditions during the dark phase of the day, in which mice are more active, and during the light phase. The increase in basal exploratory activity found in the Y_2 KO mice when exposed to a new environment could suggest a reduced anxiety-like state, given that the exploratory behaviour is related to a less anxious state of the animals (Fig. 7). The same analysis was performed on the Y_2 KO and WT female mice, but no changes were found, underlining basal gender differences. This gender behavioural variability is not surprising: it has been previously described that the behaviour is clearly influenced by endocrine factors, differing between genders (Lathe, 2004). Moreover, gender has been demonstrated to influence both baseline behavioural characteristics (Voikar *et al.,* 2001) and antidepressant responses in different strains of mice (Caldarone *et al.,* 2003).

In the present study, both male and female Y_2 KO and WT mice were also exposed to the forced swim test (FST), a well established research tool to investigate the depressive-like state of the animals, but no difference in terms of immobility time was found between the two groups in both genders (Fig. 11). Immobility in the FST is considered a marker of the depressive-like behaviour (Porsolt *et al.,* 1977), meaning that in this study using pure

background C57BL/6 mice the deletion of the Y_2 gene seemed not to induce a depressive-like state. However, previously published data on the same mice, in which the initial mixed 129SvJ-C57BL/6 genetic background was maintained, have reported a significant reduction of the immobility time in the Y_2 KO male mice compared to their control WT, suggesting a reduced depressive-like state in the mice lacking Y_2 receptor (Tschenett *et al.,* 2003). To further analyze the depressive-like behaviour of the pure background male and female Y_2 KO and WT mice, a possible differential effect in terms of immobility time in the FST was evaluated after acute administration of desipramine, a frequently used antidepressant acting as a norepinephrine-transporter inhibitor, inducing a reduction of the immobility time in the FST, counteracting the depressive-like state. The same response to the pharmacological treatment was observed in the two groups of mice, consisting on a reduction of the immobility time after the administration of the higher dose of desipramine in the Y_2 KO and WT male mice (Fig. 9) and of the lower dose in the female mice (Fig. 10). These results could suggest that the lack of Y_2 receptor is not interfering with the action of the antidepressant or with the functioning of the norepinephrine system, in which desipramine acts, although also in this case the activation of a compensatory mechanism can not be excluded.

The anxiety-related behaviour of the Y_2 KO and WT mice was also assessed, exposing them to the elevated plus maze (EPM), a validated test of anxiety, in which the animals have to choose between their natural aversion for open spaces and their drive to explore a novel environment (Pellow & File, 1986). This test confirmed the lack of significant behavioural differences between Y_2 KO and WT mice: they spent comparable times in the open and closed arms of the maze and a comparable number of entries in the open arms was also recorded (Fig. 8). However, previously published data on the same mice, in which the initial mixed genetic background has been maintained, have showed a differential anxiety-related behaviour between the Y_2 KO and WT mice when exposed to the EPM, with a significant increase of the time spent in the open arms of the maze observed in the mice lacking Y_2 receptor, thus displaying a less anxious behaviour compared to the WT mice (Tschenett *et al.,* 2003; Redrobe *et al.,* 2003). Since no baseline differences were detected in this study, the EPM was also performed after challenging the animals with an acute stress, but also in this case, a similar performance in the EPM was reported by the two groups of animals (Fig. 8).

Finally, the basal levels of two stress hormones, ACTH and corticosterone, in the pure background Y_2 KO and WT mice were also characterized, to investigate a potential

dysfunction of the HPA axis activity, but no differences were found, confirming the previous results (Fig. 12). In addition, a possible differential hormonal release in response to stress was investigated, since stress is a well known activator of the HPA axis (Smith & Vale, 2006; Herman *et al.,* 2005). Thus, the two groups of mice were exposed to an acute stress and a consequent strong release of both ACTH and corticosterone was observed, as previously described (Assenmacher *et al.,* 1995; Armario, 2006), but it was not different in the Y_2 KO and WT mice (Fig. 12). In summary, the present study seemed not to confirm the previously published data showing an involvement of the Y_2 receptor in the modulation of the depressive- and anxiety-like states (Tschenett *et al.,* 2003; Redrobe *et al.,* 2003). The results of this study were quite unexpected, given the significant behavioural differences found between the two strains of mice in the two previously published works. Thus, to confirm the deletion of the Y_2 receptor gene in the Y_2 KO mice used in this study, a series of *in vitro* investigations, such as RT-PCR, receptor autoradiography, *in situ* hybridization and RT-qPCR reactions (Figg. 13, 14, 15, 16, 17) were performed, demonstrating the lack of the Y_2 receptor in the KO mice and its presence in the WT.

The behavioural differences observed between mixed background 129SvJ-C57BL/6 mice and pure background C57BL/6 mice, although surprising, have been previously described by studies demonstrating distinct and specific differences in the anxiety-related behaviours, depressive-like state and stress responsiveness in different mouse strains, indicating that such behaviours could be influenced by the genetic component (Anisman & Zacharko, 1992; Griebel *et al.,* 2000; Anisman *et al.,* 2001; Belzung, 2001). The situation could be even more complicated, in presence of a genetic manipulation: complex interactions between genetic deletions and background strains could occur, resulting in phenotypic differences (Carlson, 1997; Cook *et al.,* 2002; Lesch *et al.,* 2003). In this particular case, a variety of studies on C57BL/6 mouse strain have demonstrated higher levels of basal immobility in the FST and in the tail suspension test, another behavioural tool to evaluate the depressive-like state of the animals, compared to many other strains, included the 129Sv (Lucki *et al.,* 2001; Ducottet & Belzung, 2005; Jacobson & Cryan, 2005). These findings suggest that the C57BL/6 mice could be less sensitive to depressive situations (Shanks & Anisman, 1993, 1988). This hypothesis was confirmed by the results obtained in the 10 minutes FST performed in the present study and by Tschenett and collaborators. The immobility time spent by the pure background C57BL/6 WT mice in 10 minutes was around 280 seconds, whereas the immobility time spent by the mixed background 129SvJ-C57BL/6 WT mice was around 170 seconds, probably due to the presence of the 129SvJ genetic

component. Moreover, further studies, such as acute inescapable shock and chronic unpredictable stress, have demonstrated a reduced or absent behavioural sensitivity to stressful stimuli in the C57BL/6 mouse strain (Zacharko *et al.*, 1990, 1987; Pothion *et al.,* 2004; Mineur *et al.,* 2003; Ducottet *et al.,* 2004; Ducottet & Belzung, 2005). These results are consistent with the present study, in which an acute stress did not induce behavioural effects in the EPM in Y_2 KO and WT mice with pure C57BL/6 genetic background (Fig. 8). In support to the present findings, other examples of deletions of the same gene on multiple genetic background mice have showed the presence of different anxiety-related phenotypes (Ramboz *et al.,* 1998; Heisler *et al.,* 1998; Parks *et al.,* 1998). In addition, a number of strain comparisons have illustrated the impact of the genetic background on the responses to different psychopharmacological agents, sustaining a genetic-based variability also in response to specific drugs (Crawley *et al.,* 1997; Griebel *et al.,* 2000; Lucki *et al.,* 2001; Jacobson & Cryan, 2007). In particular, the relative variability of performance of mouse strains in the FST suggested that a selection of the animals strain is necessary to analyze their responses to antidepressant drugs. The least variable strain at baseline, the C57BL/6 strain, has provided the greatest precision of measuring changes in drug effects (Lucki *et al.,* 2001; Jacobson & Cryan, 2005). However, differences between strain phenotypes could not only represent a reflection of the genotypes, but the environment vs. genes interactions have been also showed to significantly influence the phenotypic behaviour (Crabbe *et al.,* 1999; Carola *et al.,* 2006). Despite careful standardizations of the protocols, variations in the experimental methodologies, even subtle ones, and in the equipment performing the experiments, that could be found between different laboratories using the same protocols, may influence the behavioural results obtained using different mouse strains (Crabbe *et al.,* 1999; Crabbe & Wahlsten, 2003). Particularly, the behavioural testing paradigms can be strongly influenced by variations in holding and test conditions, thus caution has to be used when comparing results (Jacobson & Cryan, 2007). The experimental conditions adopted in the present study when performing the behavioural tests reproduced as much as possible those performed by Tschenett and colleagues at the Innsbruck University, in terms of housing conditions, total duration of the tests, time of recording, parameters of the cylinder in the FST and of the maze in the EPM. However, as it has been demonstrated, handling could also influence the behavioural responses of different mouse strains in different laboratories (Jacobson & Cryan, 2007). To exclude these possible methodological differences between the experiments, a chort of animals was sent to Innsbruck University, where the FST and the EPM were performed at their working conditions. However, they exactly reproduced the results

obtained in this study, thus excluding a possible effect due to differences in the experimental conditions used.

Overall, the genetic background seemed to represent the unique difference existing between the mice used in these studies. Therefore, it could be possible to hypothesize that the different genetic background was, at least in part, responsible of the behavioural discrepancies found in the different laboratories. In particular, the present findings seemed to emphasize the role of the genetic background in influencing the depressive- and anxiety-related behavioural phenotypes. A possible consequence could be the genetic-related difference in the basal NPY levels between the two strains, in line with a previous work showing that a different inborn alcohol drinking behaviour between C57BL/6 and DBA/2 mice has been related to a strain different basal NPY expression in the nucleus accumbens (Misra & Pandey, 2003). In support to these findings, another report have illustrated differential basal NPY levels between the C57BL/6 and DBA/2 mouse strains also in other brain regions (Hayes *et al.,* 2005). Thus, in the present study, the potential different NPY baseline levels between C57BL/6 and 129SvJ mice in the hypothalamic area were investigated, as a possible reason to explain the different behavioural phenotypes. However, it failed in demonstrating strain differences in the basal NPY levels (Fig. 17): this could be due to the limited number of animals used or to the selection of the brain region analyzed. Further investigations using a bigger number of animals for each strain and involving some other brain regions would be necessary to complete the study.

It should also be considered that the deletion of a gene in an intact organism, such as a knockout mouse, could be followed by possible redundancy, compensations and different developmental roles of other related genes. Thus, the expression of the phenotype of the genetically modified mice may be not only characterized by the lack of a specific gene product, but by the changes that may have happened at the molecular and cellular levels in the mutant organism (Sibille & Hen, 2001). However, this line of study has been severely limited by the extent of the current knowledge of the function of the majority of genes and their implications in various biological systems and pathways. Therefore, another hypothesis to explain the lack of a differential depressive- and anxiety-related behaviour between Y_2 KO and WT mice could be the development of an adaptation to the functional absence of the Y_2 receptor in the KO mice, suggesting the possible occurrence of mechanisms of compensations by functionally related systems. The risk of the development of compensatory mechanisms is very high in such mutant mice, referred to as “constitutive” knockout, generally exhibiting a total lack of expression of the mutated gene throughout their lifetime

in all the tissues. In these mice, the mutated gene is non-functional throughout development; consequently, changes arising due to the absence of the gene for the entire life span of the mouse could yield unexpected phenotypes in mature animals. In this specific case, the lack of the Y_2 receptor gene during maturation of the mice could have interfered with the normal developmental program of the organisms, or could have induced changes in the other systems to compensate its absence (Gingrich & Hen, 2000). This problem could be avoided using "conditional" or "inducible" knockout mice, in which a targeted gene is not absent during the development of the organism, but its expression is controlled in a temporal and/or regional way, reducing the probability of adaptations (Sibille & Hen, 2001; Stark *et al.,* 1998).

The use of other methodologies aimed at manipulating gene expression could be suggested, such as the short-interfering RNA, in which double-stranded RNAs injected in the animals cause post-transcriptional silencing of gene expression (Thakker *et al.,* 2004). Moreover, this methodology can be applied on adult animals, avoiding the risk of possible developmental compensations and rats can also be used, preferred than mice because they present less behavioural variability in the different strains. However, studies involving animals treated with selective antagonists acting on specific targets would be considered the most specific indication of the role of such biological targets in the pathophysiology of a specific disease. Unfortunately, the lack of selective, brain penetrant compounds targeting the NPY receptors available for *in vivo* studies leads to the use of other less precise research tools.

Overall, the present investigation gave further supporting evidences to the importance of using specific mouse strains in the behavioural studies, especially when focusing on the emotional-related behaviours.

5.2 ANIMAL MODELS OF DEPRESSION

The previously described behavioural characterization of the Y_2 receptor KO mice was not conclusive for the understanding of the Y_2 receptor role in mood disorders; however, a different experimental approach was then considered, consisting on the use of animal models of depression. These models are considered a potential investigative tool to provide insights into the pathophysiology of depression, to investigate the mechanism of action of conventional antidepressants and to support the discovery of new antidepressant drugs; however, they have not been extensively studied with regard to altered neuropeptide expression (Willner, 1997b). In the present study, they were used to investigate the possible transcriptional changes of the NPY system in depressive disorders. The choice of considering different animal models was due to the difficulties in representing with animals a psychiatric disease, which is a typical human condition. In fact, an animal model of depression can only be considered a simplified representation of a condition similar to human depression, not exactly reproducible with animals.

5.2.1 Flinders Sensitive Line rats

Numerous studies have demonstrated that the Flinders Sensitive Line (FSL) rats constitute a relevant animal model of depression, which mimics many of the biological and behavioural characteristics associated with depression in human subjects, which become even more evident in the FSL rats after exposure to stressors (Overstreet *et al.,* 2005; Pucilowski *et al.,* 1993).

Many findings focusing on neuropeptide systems have suggested that the FSL rats may be considered a validated animal model for exploring the involvement of these systems in depressive disorders (Owens et al., 1991; Husum et al., 2008, 2003, 2001; Mathé *et al.,* 2007).

In the present study, a significant baseline difference between the FSL and FRL rats in the dentate gyrus of the hippocampus was demonstrated, with lower NPY mRNA expression levels in the “depressed” FSL rats compared to their control FRL (Fig. 18). This result is consistent with a number of studies providing supporting evidences for a possible role of the NPY system in the mechanisms underlying the pathogenesis of depression. In particular, the existence of an alteration of the NPY system in this hippocampal region of the FSL rats has been previously demonstrated by Bjørnebekk and collaborators (2006), who have showed a lower basal NPY mRNA expression in the dentate gyrus of the FSL rats,

whereas in other studies the FSL rats have reported a reduced NPY mRNA expression in the CA hippocampal region (Caberlotto *et al.,* 1998a) and lower NPY-LI in the whole hippocampus, compared to their control FRL (Caberlotto *et al.,* 1999; Husum *et al.,* 2008, 2001; Jimenez Vasquez *et al.,* 2000 a,b; Wortwein *et al.,* 2006). The reason for these discrepancies in the different hippocampal subregions is not clear; it could be due to the fact that the rats used in the above mentioned experiments were exposed to different treatments. Nonetheless, the hippocampal formation seems to be an important region related to NPY and depressive disorders, since in another genetic animal model of depression, the Fawn Hooded rats (Mathé *et al.,* 1998), as well as in environmental models, such as maternal separation or chronic mild stress models (Husum & Mathé, 2002; Husum *et al.,* 2002; Jimenez-Vasquez *et al.,* 2001; Sergeyev *et al.,* 2005), reduced hippocampal NPY has been found. The hippocampus is a brain region with a high functional and structural plasticity, which has been demonstrated to be important for learning and memory processing (Gould *et al.,* 1999 a,b; Lisman, 1999) and it is one of the few structures in the adult brain where new neurons are formed (Altman, 1962). Hippocampal neurons are sensitive to stress and pre-clinical studies have indicated that stress may cause atrophy and death of the pyramidal neurons in the hippocampus, as well as decreased neurogenesis in the dentate gyrus (McEwen *et al.,* 1992; McEwen & Magarinos, 1997; Gould & Tanapat, 1999). Moreover, it has been postulated that depressive symptoms are associated with reduced hippocampal plasticity and decreased neurogenesis (Duman *et al.,* 1999); furthermore, a decreased hippocampal volume has been observed in the depressed patients and the degree of the hippocampal volume reduction has been correlated with the duration of the disorder (Sheline, 1996; Sheline *et al.,* 1999, 1996; Bremner *et al.,* 2000; Campbell & Macqueen, 2004; Campbell *et al.,* 2004). In contrast, treatments inducing an antidepressant effect, such as antidepressant drugs (Malberg *et al.,* 2000), electroconvulsive treatments (Nibuya *et al.,* 1995; Hellsten *et al.,* 2002) and physical exercise (Neeper *et al.,* 1996; van Praag *et al.,* 1999 a,b; Bjørnebekk *et al*., 2005) could induce an increased hippocampal neurogenesis. However, different hypothesis debating on the key role of hippocampal neurogenesis or hippocampal atrophy in depression, as well as on the effect of antidepressants on cell proliferation have been proposed, suggesting that neurogenesis plays a role in depression, but neither this phenomenon is likely to be exclusive for the depressive disorders, nor it can explain all the clinical symptoms of depression, or all the effects induced by antidepressant drugs (Steckler & Prickaerts, 2004). Recently, NPY has been demonstrated to be involved in the adult hippocampal neurogenesis in the dentate gyrus, inducing an increase of the hippocampal cell number in the hilus and in the subgranular zone,

thus promoting cell proliferation (Howell *et al.,* 2005). In addition, running has been demonstrated to induce a marked NPY mRNA increase in the CA4 and dentate gyrus of the hippocampus in the FSL rats compared to the FRL (Bjørnebekk *et al.,* 2006). Moreover, the running-induced increase of NPY mRNA in the FSL rats has been strongly correlated with the running-induced increase of cell proliferation (Bjørnebekk *et al.,* 2005), sustaining that NPY is one of the factors triggering cell proliferation. Consistent with these studies, reasoning is the finding that NPY increases adult neurogenesis in the olfactory bulb (Hansel *et al.,* 2001). In view of these data and of the evidence suggesting that a decreased neurogenesis, with a consequential hippocampal atrophy, leads to a depressive state (Fuchs *et al.,* 2004), the lower levels of the NPY mRNA expression found in baseline conditions in the dentate gyrus of the FSL rats could represent the biological correlate of their depressive-like behaviour. Moreover, the reduced NPY levels found in the "depressed" rats are in line with clinical findings, demonstrating a decrease of NPY in the cerebrospinal fluid (CSF) (Gjerris *et al.,* 1992; Heilig *et al.,* 2004; Widerlov *et al.,* 1988) as well as in the plasma (Nilsson *et al.,* 1996; Hashimoto *et al.,* 1996) and in selected brain regions (Widdowson *et al.,* 1992) of depressed patients.

Unexpectedly, in the present study, no significant changes were found in the arcuate nucleus of the hypothalamus, even if the NPY system in this brain region has been demonstrated to be involved in the pathophysiology of depression and in the action of antidepressant drugs (Baker *et al.,* 1996; Makino *et al.,* 2000; Kim *et al.,* 2003; Sergeyev *et al.,* 2005). In fact, an earlier study has reported basal strain differences in the NPY mRNA expression between the FSL and FRL rats in this region (Caberlotto *et al.,* 1998a).

Although the expression of NPY has been largely studied in the FSL rats, less is known about the receptors. In the present work, the mRNA expression of the three major NPY receptor subtypes, Y_1, Y_2 and Y_5, was analyzed in the FSL and FRL rats, in baseline conditions and after exposure to an acute stress. Only the Y_5 receptor mRNA expression was inversely regulated by the stress exposure in the central amygdala of the two rat strains: the Y_5 mRNA levels were increased by stress in the FRL rats, while a stress-induced reduction was observed in the FSL group (Fig. 19), suggesting that the "depressed" FSL rats have an altered response to stress compared to their controls. Interestingly, a recent study focusing on the CRF mRNA expression in the same animals has found a differential response to stress in the two rat strains in the same nucleus of the amygdala, in which the FRL stressed rats have displayed increased CRF mRNA, whereas the FSL rats have not reported alterations, showing a blunted response to stress (Zambello *et al.,* 2007). Thus, these results could

suggest the existance of an anatomical and functional interaction between the NPY and CRF systems in a brain area involved in the regulation of emotionality, as previously demonstrated (Sajdyk *et al.,* 2004; Krysiak *et al.,* 2001; Britton *et al.,* 2000; Heilig *et al.,* 1994), sustaining the hypothesis that they exert a reciprocal and opposite regulation of responsiveness to stressful stimuli, as also demonstrated in other brain regions (Heinrichs *et al.,* 1992; Palkovits, 1999; Morris & Pavia, 1998). On the contrary, the other two receptor subtypes considered in the present study, Y_1 and Y_2, did not display differences in baseline conditions or after exposure to stress. A series of studies reporting alterations of the Y_1 receptor in the FSL rats (Caberlotto *et al.,* 1998a; Jimenez-Vasquez *et al.,* 2007) could have led to suggest the Y_1 receptor as the subtype mediating the actions of NPY on the emotional processes. This was not confirmed in the present investigation and these discrepant results could be due to the different experimental procedures applied: in the previous works, the animals have been injected with fluoxetine or vehicle for 14 days (Caberlotto *et al.,* 1998a), or have received a series of ECS or Sham (Jiménez-Vasquez *et al.,* 2007), while in this study rats were not treated, but only exposed or not to an acute stress. In support for the notion that these discrepancies could be due to a differential experimental protocol, Husum and colleagues (2003) have demonstrated that repeated handling, alone or associated with injections of a drug, has respectively increased hypothalamic NPY-LI and reduced hippocampal NPY-LI of both FSL and FRL rats.

Focusing on the Y_2 receptor, no alterations of the binding sites have been reported in the FSL rats, even after the antidepressant treatment (Caberlotto *et al.,* 1999). Moreover, the absence of changes in the expression of Y_2 receptor mRNA was in line with previous studies on "depressed" rats (Caberlotto *et al.,* 1998a, 1999) and it could lead to hypothesize a less important role of this receptor subtype in depression. In addition, this lack of changes on Y_2 receptor mRNA expression has been demonstrated to be in line with human findings, comparing the Y_2 mRNA expression of subjects affected by bipolar disorder and major depression with normal controls in the prefrontal cortex (Caberlotto *et al.,* 1999, 2001), temporal cortex and amygdala (section 5.3).

5.2.2 Chronic mild/unpredictable stress

The chronic mild/unpredictable stress (CMS) is a validated animal model of depression, characterized by a long and complex experimental procedure. However, the chronicity of the model also represents one of its major strengths in reproducing the stress conditions of human life.

The present study focused on the possible effects of the CMS procedure on the NPY system, to provide evidences of transcriptional changes in discrete brain regions related to emotions, stress and defense reactions. Only the Y_5 receptor displayed a significant effect of the exposure to the CMS, consisting on a stress-induced reduction of the Y_5 receptor mRNA expression in the cingulate cortex, medial amygdala and in the arcuate hypothalamic nucleus (Fig. 22). These alterations of the Y_5 receptor mRNA expression in the CMS model in brain regions known for their fundamental role in mood disorders, together with the previously described results on the FSL rats model of depression (section 5.2.1), supported the role for this NPY receptor subtype in the regulation of the depressive-like state and its involvement in the emotional responses to stress.

Surprisingly, the present study did not evidence any effect of the CMS exposure on the NPY levels (Fig. 20), whereas a previous study has demonstrated higher NPY mRNA levels in the arcuate hypothalamic nucleus and lower levels in the hippocampal dentate gyrus of Wistar rats exposed to the CMS compared to the non-stressed (Sergeyev *et al.,* 2005). However, an immunohistochemistry study has showed that the exposure to the CMS induced a reduction of the NPY protein expression in several hypothalamic and thalamic areas, including the arcuate nucleus of hypothalamus, known for its critical role in the stress responses (Thorsell *et al.,* 1998; Kim *et al.,* 2003). However, the stress protocols applied in these different studies were not exactly the same, both in terms of duration of the stress procedure and in terms of kinds of stress to which the animals were exposed; this could have significantly influenced the final results, since another study has also demonstrated that even handling can induce considerable differences in the expression levels (Husum *et al.,* 2003). Thus, contrasting results on the effects of the CMS have been reported, since it represents one of the most variable and difficult experimental stress protocols. The lack of changes on the NPY levels showed in the present study after the chronic stress procedure could be due to the possible activation of a mechanism of adaptation to the stress condition, as demonstrated in some preclinical and clinical studies (Chen & Herbert, 1995; Martinez *et al.,* 1998; Thorsell *et al.,* 1999; McEwen & Seeman, 1999; McEwen 2007, 2006). Moreover, a recent study has reported that the effects of stress could be frequently experienced even several weeks following the exposure to the stressor (Matuszewich *et al.,* 2007). In support to this hypothesis, previous research works on chronic stressed animals have measured increased anxiety levels 1-3 weeks following the last stress exposure (Adamec, 2003; van Dijken *et al.,* 1992; Koolhaas *et al.,* 1997; Koba *et al.,* 2001; Buwalda *et al.,* 2005). This hypothesis is consistent with evidences demonstrating that the unpredictable stress in rodents has not

caused behavioural effects immediately after the cessation of the stress procedure (D'Aquila *et al.,* 1994; Vyas *et al.,* 2002). However, in the present study the effects of the CMS on the NPY expression levels were evaluated only 24 hours after the cessation of the chronic stress procedure, thus a time-course study could be important to address this issue. Moreover, it has been showed that sensitivity to the CMS varies between strains and interacts with genetic factors relevant to depression: for example, the putative "depressed" FSL rat strain has been demonstrated to be more susceptible to the CMS-induced anhedonia than the control FRL rats, indicating that the "depressed" strain appears more prone to the effects of stress (Pucilowski *et al.,* 1993). Thus, as previously discussed, an evaluation of the characteristics of the rat strain in terms of predisposition to stress, or to anxiety and depression would be appropriate before starting this kind of experimental procedure.

In conclusion, these findings suggest that there could be individual differences in the activity of the NPY system, which may determine personal reactions to stressful situations, which may also lead to different compensatory mechanisms providing protection from stress. Thus, in general, it would be appropriate to systematically investigate the effects of the CMS at different time points following the stress exposure, performing a time-course study of the effects of stress.

The Y_1 and Y_2 receptors mRNA expression were not affected by the exposure to the CMS (Fig. 21), possibly explained with the occurrence of mechanisms of compensations by functionally related systems. Unfortunately, studies investigating these issues are not yet available; thus, for this reason, further studies are needed to expand the knowledge on the mechanisms regulating the role of these receptors on mood disorders.

The present study has also considered the differential basal anxiety levels of the rats, based on their behavioural response to an initial EPM test, thus dividing them in two groups defined low anxiety (LA) and high anxiety (HA). Successively, some animals of each group were exposed to the CMS protocol, while the controls were subjected to daily handling; at the end of the stress procedure, another EPM was performed to assess their final anxiety levels. The present study provided evidences of possible basal transcriptional differences of the NPY system between the two groups of rats in baseline conditions, based on their different anxiety levels. A significant difference of the NPY mRNA expression between the two groups of rats in baseline conditions, with higher expression levels in the HA animals compared to the LA, was observed in the medial nucleus of the amygdala (Fig. 20). This result is particularly interesting in view of the well known anxiolytic-like properties of NPY (Heilig *et al.* 1993, 1992, 1989; Wahlestedt *et al.,* 1993; Britton *et al.,* 1997; Kask *et al.,*

1998a) and of the key role of the amygdala in the regulation of the affective behaviours, with a strong involvement in anxiety, fear and emotional memory (Aggleton, 1993; Gallagher & Chiba, 1996). In addition, this finding is in line with previous works, assessing that the NPY role in the amygdala has been consistently associated with anxiety; in particular, NPY injections in this region induced anxiolytic-like effects (Heilig *et al.* 1995, 1993). Therefore, increased NPY levels in this brain region of the HA rats could represent a protective mechanism, or a possible activation of compensatory processes, such as an attempt to control the high anxiety-like state of these rats, restoring a normal condition. Furthermore, in support to the present findings, a study comparing the NPY levels between the HA Fischer-344/N rat strain and the LA WAG/G strain in baseline conditions has reported higher NPY brain levels in the HA strain (Sudakov *et al.,* 1999).

In the present study, a significant difference in the Y_1 receptor mRNA levels between the HA and LA rats was observed in the hippocampal dentate gyrus, in which the HA rats displayed lower Y_1 mRNA levels compared to the LA in baseline conditions (Fig. 21). This result could support the hypothesis of the role of the Y_1 receptor in mediating the NPY anxiolytic-like activity (Kask *et al.,* 2002), in line with evidences demonstrating that the Y_1 receptor agonists produce NPY-like anxiolytic effects (Heilig *et al.,* 1993; Broqua *et al.,* 1995; Britton *et al.,* 1997), whereas the antagonists are anxiogenic (Wahlestedt *et al.,* 1993a; Kask *et al.,* 1998a, 1996). In this case, the higher Y_1 mRNA expression in the LA rats could be reflected in their behavioural phenotype, inducing a reduction of their anxiety-like state, thus further sustaining the Y_1 receptor involvement in the mechanisms regulating anxiety. This result is in line with the findings on the FSL rats, in which lower Y_1 mRNA levels were found in the dentate gyrus of hippocampus in baseline conditions compared to the FRL (Caberlotto *et al.,* 1998a; Jiménez-Vasquez *et al.,* 2007), suggesting that altered levels of the Y_1 receptor in this brain region could be related to the development of the anxiety-like state and depressive disorder. An opposite result was observed in the central amygdala, in which the HA rats showed higher Y_1 mRNA levels compared to the LA animals (Fig. 21), confirming the hypothesized role of the Y_1 receptor in the amygdala in the mechanisms regulating the anxiety-like state (Sajdyk *et al.,* 1999; Kask *et al.,* 2002, 1998b, 1996; Primeaux, 2005); in particular the central nucleus of this brain region is known to be involved in the behavioural, endocrine and autonomic responses to stress and anxiety (Davis *et al.,* 1994). Thus, an increased expression of Y_1 receptor mRNA in this nucleus of the amygdala in the HA animals could be responsible for their anxious phenotype. However, this result seems not to be in line with a previous work of Heilig and colleagues (1993),

demonstrating that the administration of Y_1 receptor agonists led to the activation of the Y_1 receptors in the central amygdala, inducing anxiolytic effects. Finally, it is interesting to observe that, in baseline conditions, the same receptor can be oppositely regulated in the HA and LA rats in two different brain areas, underlying the regional specificity of the functions of these receptors.

Regarding the Y_2 receptor mRNA expression, no significant changes between HA and LA rats were observed in baseline conditions, consistently with the previously discussed findings on this receptor in the FSL and FRL rats (section 5.2.1) and with other studies on the FSL rats (Caberlotto *et al.,* 1999, 2001). Although the lack of changes in the Y_2 receptor mRNA expression in the depressive disorders is in line with human findings (Caberlotto *et al.,* 1999, 2001), the role of the Y_2 receptor in the regulation of anxiety has been supported by the findings on Y_2 KO mice (Tschenett *et al.,* 2003; Redrobe *et al.,* 2003), or by studies investigating the activity of compounds acting selectively on the Y_2 receptor tested in animal models of anxiety (Sajdyk *et al.,* 1999; Nakajima *et al,* 1998; Bacchi *et al.,* 2006). Hopefully, further studies will help to clarify the mechanisms regulating the role of this receptor in mood disorders.

5.2.3 Chronic social defeat stress in rat and tree shrew

The impact of stressful events on the development of psychopathologies has been largely investigated in pre-clinical studies, however the purpose of studying the animal responses to stress consists on clarify the human condition, in which the most common stressors have been demonstrated to have psychological or social nature (Kessler *et al.,* 1985; Kessler, 1997). Therefore, the use of social conflict between members of the same species to generate stress has advantages over animal models requiring aversive physical stimuli. A number of studies have showed that subordination stress, or social defeat, is a natural stressor leading to a variety of long-lasting physiological, behavioural and molecular changes (Bjorkqvist, 2001; Fuchs & Flugge, 2002). In particular it can affect reward-related processes (Von Frijtag *et al.,* 2000) and evoke anhedonia and motivational deficits (Rygula *et al.,* 2005), as well as changes in neurotransmitter release in different brain areas (Isovich *et al.,* 2001; Krugers *et al.,* 1993; McKittrick *et al.,* 2000; Fuchs & Flugge, 2002). Moreover, a number of evidences have reported that these changes were reversed by treatments with antidepressant drugs (Fuchs *et al.,* 1996; Kramer *et al.,* 1999; Fuchs *et al.,* 2004; Rygula *et al.,* 2006), confirming that the chronic social defeat stress represents a validated animal model of depression.

Based on these premises, the present study investigated any potential transcriptional

changes of the NPY system in two different animal species chronically exposed to social defeat stress and the possible effect of fluoxetine, a classic SSRI antidepressant, to clarify the mechanisms by which chronic stress acts on this neuropeptidergic system, presumably inducing a depressive-like state, and to assess if this system is involved in the mechanism of action of the antidepressant drugs. Focusing on the rat, the NPY mRNA levels were significantly decreased in the CA3 hippocampal region of the stressed group of animals compared to the controls (Fig. 23), hypothesizing a possible depressive-like effect of the chronic social stress, in view of the well known antidepressant properties of NPY in animals (Heilig, 2004). This result correlated with previous findings in the FSL rats model of depression, in which the "depressed" animals have displayed reduced baseline levels of NPY mRNA in the CA3 hippocampal region (Bjørnebekk *et al.,* 2007), as well as reduced NPY-LI in the CA1-2 region (Jimenez-Vasquez *et al.,* 2007), or in the total CA region (Caberlotto *et al.,* 1999, 1998a) compared to the FRL control. Moreover, the present study showed an opposite regulation between the stress exposure and the antidepressant treatment in the dentate gyrus of the hippocampus, in which a down-regulation of the NPY mRNA expression was observed in the stressed rats compared to the controls; however, the chronic administration of fluoxetine reversed the effect of stress, restoring control NPY levels (Fig. 23). This reduced NPY mRNA expression in the dentate gyrus of the chronically stressed animals was in line with many other studies on various animal models of depression (Caberlotto *et al.,* 1998a; Mathé *et al.,* 1998; Husum and Mathé, 2002; Bjørnebekk *et al.,* 2006; Jimenez-Vasquez *et al.,* 2007) and together with the previously mentioned NPY reduction in the CA3 region, it supported the hypothesis that diminished hippocampal NPY levels seemed to be strictly related to a depressive-like state of the animal (Heilig, 2004). The hippocampus has been demonstrated to play an important role in the emotional processing and in the key aspects of learning and memories (Eichenbaum *et al.,* 1992; Squire 1992). Moreover, it has been showed to belong to a neuroendocrine circuit, the limbic-hypothalamic-pituitary-adrenal (LHPA) system, in which limbic and hypothalamic brain structures integrate emotional, cognitive and autonomic inputs, determining the responses to stressful experiences (Herman *et al.,* 1996; Lopez *et al.,* 1999). In addition, the hippocampal formation has been demonstrated to express a high concentration of glucocorticoid receptors (de Kloet *et al.,* 1998) and to modulate glucocorticoid release, exerting inhibitory effects via a negative feedback on the HPA axis (Jacobson & Sapolsky, 1991; Herman, 1993). While short-term activation of this circuit is essential for vital functions, a constant hyperactivity of the circuit induced by chronic stress, resulting in chronically elevated glucocorticoids, has

been showed to lead to deleterious effects (Herman, 1993), such as hippocampal neuronal loss (Sapolsky, 1986). It has been observed that chronic stress impaired the feedback mechanisms, associated with decreased gene expression of hippocampal corticoid receptors (Meyer *et al.,* 2001). Moreover, increasing evidences have suggested a connection between disturbances of the activity of the LHPA system with both structural alterations and volume reduction of the hippocampal formation, leading to cognitive or memory impairments (Sheline, 1996; Lupien *et al.,* 1998). The glucocorticoids have been considered responsible of the deficits in cognitive and memory function, via significant atrophy of the hippocampal CA3 neurons, caused by a reduction of the dendritic arborizations and a loss of the dendritic spines; moreover, the gluococorticoids induced a reduction of the granule cell layer and of the subgranular zone of the hilus of the dentate gyrus, as well as of the whole hippocampal volume (Wooley *et al.,* 1990). This hippocampal neuronal loss has been demonstrated to be mediated by apoptotic mechanisms (Sapolsky 1985 a,b) or by inhibition of the hippocampal neurogenesis (Magarinos *et al.,* 1996; Gould *et al.,* 1997), observable with brain imaging studies on human patients, which have reported a consequential deficit of the cognitive functions (McEwen *et al.,* 1997; McEwen & Seeman, 1999). These findings seemed to hypothesize that impairment of brain structural plasticity represents an important feature of depressive disorders (Duman *et al.,* 1999). Finally, various classes of antidepressant have been demonstrated to counteract the reduction of the dendritic arborizations of the CA3 hippocampal neurons (Norrholm & Ouimet, 2001) and to activate the neurogenesis in the DG (Malberg *et al.,* 2000), supporting the hippocampal hypothesis of depression. Based on these evidences, the present findings showing reduced NPY mRNA expression in the hippocampal CA3 region and dentate gyrus of the stressed rats could be related to the loss of hippocampal neurons under extreme, prolonged stress exposure, associated to a glucocorticoid excess. A parallel study conducted on rats exposed to the same protocol of those used in this work supported the hypothesized theory demonstrating an inhibited dentate gyrus neurogenesis after exposure to chronic psychosocial stress, reversed by chronic fluoxetine treatment (Czéh *et al.,* 2007). Moreover, the present study gave further support to the previously mentioned anatomical and functional link between hippocampus and hypothalamus: all the changes affecting the NPY receptors were observed in hypothalamic nuclei. In particular, Y_1 and Y_5 receptors displayed a reciprocal regulation between stress exposure and fluoxetine treatment in the ventro-medial-hypothalamus, with reduced transcriptional levels in the stressed group of rats compared to the controls, whereas the mRNA levels of the stressed animals treated with chronic fluoxetine were significantly up-regulated to the control levels, underlying that

fluoxetine counteracted the stress-induced effects (Figg. 24, 26). Fluoxetine, a selective serotonin re-uptake inhibitor (SSRI), was chosen in the present study because it represents one of the most used antidepressants in clinic. Its activity in restoring normal expression levels altered by chronic stress displayed both in NPY and in the two receptor subtypes represented the most relevant result obtained in this study. The purpose of the present experiments consisted on mimicking a realistic situation of an antidepressant intervention in animal studies; therefore, the drug was administered orally for 4 weeks, a clinically relevant time period and the plasma concentrations of the antidepressant were similar to those effective in clinical studies. In fact, parallel studies of the physiological parameters (Rygula *et al.,* 2006; Czéh *et al.,* 2007) have confirmed that the plasma concentration of fluoxetine resulted comparable in the control and stressed groups of rats and that the concentrations of fluoxetine in the plasma were in line with therapeutically effective doses (Baumann *et al.,* 2004). Moreover, the pharmacological treatment was applied while exposing the animals to the stress procedure and the stressful influences were continuously present during the whole period of treatment in order to mimic the human condition.

In conclusion, the present investigation further validated the chronic psychosocial stress model as a predictive animal model of depression, giving further indications of the involvement of the NPY system in stress responses and depressive disorders, demonstrating the involvement of the NPY system in the activity of the antidepressant drugs.

The reduced Y_1 receptor mRNA levels in the stressed group of animals gave further support to the above mentioned role of the Y_1 receptor in modulating the NPY functions in the stress-related responses and depressive-like states (section 5.2.2). Moreover, the similar effects observed in the Y_1 and Y_5 receptors were consistent with the hypothesis of their anatomical and functional interactions. An immunohistochemistry study has demonstrated that they co-localize in several rat brain regions (Wolak *et al.,* 2003), an *in situ* hybridization study has showed that they have an overlapping mRNA distribution pattern (Parker & Herzog, 1999) and a high dimerization state of these two receptor subtypes has been observed (Dinger *et al.,* 2003), explaining why Y_1 and Y_5 receptors are often involved in some identical functions, potentially exerting a reciprocal co-regulation (Lin, 2004; Gehlert, 2004; Gehlert *et al.,* 2007). Moreover, unlike the other NPY receptor subtypes, Y_1 and Y_5 human genes were found in close proximity to each other and were likely evolved from a unique precursor, through a gene duplication event (Herzog *et al.,* 1997).

Finally, a significant reduction of the Y_2 receptor transcriptional levels was observed in the ventro-medial hypothalamic nucleus of the stressed rats (Fig. 25), suggesting that the

chronic psychosocial conflict could be linked to alterations of the HPA axis (Kramer *et al.,* 1999; Fuchs *et al.,* 2001). Moreover, the altered HPA axis activity was supported by a parallel investigation of the physiological parameters measured on these rats, showing that the exposure to chronic social stress has induced increased adrenal glands weight (Rygula *et al.,* 2006), indicative of the effects of stress (Sapolsky *et al.,* 2000), in line with a previous study reporting increased adrenal weight following chronic mild unpredictable stress (Muscat & Willner, 1992). In this case, increased adrenal weight in the stressed animals might reflect a hyper-activation of the HPA axis, involved in the mechanisms leading to depressive states (Kramer *et al.,* 1999; Fuchs *et al.,* 2001). Moreover, the dysregulation of the HPA axis represents one of the best replicated findings in human depressed patients (Rubin *et al.,* 1987), further confirming the validity of the model. However, Rygula and colleagues (2006) have failed to demonstrate an effect of fluoxetine in reducing adrenal glands weight in these animals.

Furthermore, the present study examined the effects of the chronic psychosocial stress on the NPY system in a non-rodent species, the tree shrew (*Tupaia belangeri*), phylogenetically regarded as an intermediate between insectivores and primates, showing a high genetic homology with humans (Martin, 1990), a pronounced territoriality in male animals and a day-active life (Fuchs *et al.,* 1996). Thus, the chronic defeat stress on the tree shrews could represent a suitable and naturalistic experimental paradigm to study the mechanisms of major depression (Fuchs *et al.,* 1996). In fact, numerous behavioural and endocrine studies of the effects of chronic social stress in the tree shrew have supported this hypothesis, demonstrating that these animals resemble symptoms of human depressed patients and respond to chronic antidepressant treatments, leading to an improvement of the symptoms. Moreover, these studies have showed that chronic stress induced alteration of brain metabolism, hippocampal volume and reduction of the hippocampal neurogenesis, leading to memory and cognitive impairments, generally prevented by treatments with various antidepressants (Fuchs *et al.* 2004, 2001, 1996; Kramer *et al.* 1999; Fuchs & Gould 2000; Czeh *et al.* 2007, 2001; Fuchs & Flugge 2003, 2002; Van Kampen *et al.* 2002; Lucassen *et al.* 2004; Fuchs 2005).

Differently from the results found in rat, the present study demonstrated that a chronic exposure to defeat stress in the tree shrews induced an increase of the NPY mRNA levels in the CA1-2 hippocampal regions and in the arcuate hypothalamic nucleus (Fig. 28). The transcriptional changes, in particular those observed in the hippocampal formation, could support the hypothesis of a neuroproliferative role of NPY in the hippocampal cells (Howell

et al., 2007, 2005, 2003), representing an attempt to counteract the reduced hippocampal neurogenesis in the dentate gyrus observed in the stressed animals in a parallel study (Prof. Fuchs, personal communication). Thus, NPY has been showed to contribute to the maintenance of the hippocampal neurogenesis, suggesting a possible neuroprotective function against the stress-induced cellular damage in the hippocampus (Howell *et al.,* 2007, 2005, 2003, 2002). Thus, the hippocampal neuroproliferative role of NPY could be considered a possible mechanism by which NPY exerts its antidepressant properties and its improvement of learning and memory processing. Moreover, the alteration of the NPY expression found in the hypothalamic region, related to the alterations observed in the hippocampus, gave further support to the existence of an intimate connection between these two brain regions (Herman *et al.* 1996; Fuchs & Flugge 2003).

Focusing on the receptors, consistently with the alterations of the NPY transcriptional levels, the Y_2 receptor mRNA expression was increased in the stressed tree shrews compared to the non-stressed (Fig. 29), thus supporting the hypothesis of the activation of the NPY system against the stress-induced cellular damage in the hippocampus (Howell *et al.,* 2007, 2005, 2003).

Furthermore, the Y_5 receptor mRNA expression was found to be altered in various brain regions of the tree shrews, with a reduction of the transcriptional levels in the CA1-2 hippocampal regions in the stressed animals and a restoring of the expression levels of the control animals after treatment with fluoxetine (Fig. 30), as observed in the rats. Furthermore, in the medial amygdala and cingulate cortex, a Y_5 receptor up-regulation induced by stress exposure was found, which was not reversed by fluoxetine (Fig. 30). All these changes, together with the results obtained in the present study in other animal models of depression (sections 5.2.1 and 5.2.2) gave support for the involvement of the Y_5 receptor in the mechanisms regulating the responses to stress and in the depressive states, resulting particularly interesting since the Y_5 receptor has been mostly studied for its involvement in the mechanisms related to food intake and obesity (Gehlert, 1999; Cabrele *et al.,* 2000).

In conclusion, this study underlined that the transcriptional levels of the NPY system were affected by the chronic psychosocial stress in rat and tree shrew, showing that in some specific cases, both the species demonstrated transcriptional changes induced by fluoxetine treatment, which reversed the effects of stress by restoring the mRNA levels observed in the control subjects. These findings supported some previous evidences demonstrating that the levels of the NPY mRNA expression and the NPY-LI have been increased both in rat and human after a chronic administration of antidepressants (Heilig *et al.,* 1988; Weiner *et al.,*

1992; Nikish *et al.,* 2005) or after ECS and ECT, procedures used to treat depression in pharmacotherapy-resistant cases (Jimenez Vasquez *et al.,* 2007, 2000 a,b; Mathé, 1997, 1996, Mikkelsen *et al.,* 1994; Stenfors *et al.,* 1992; Wahlestedt *et al.,* 1990), suggesting that the antidepressive treatment modalities may act through a mechanism that increases the endogenous NPY levels. However, in this study, the stress exposure generally induced a reduction of the NPY and its receptors mRNA expression in the rat brain, whereas an opposite direction of changes was found in the tree shrew transcriptional levels, which were generally increased by stress and diminished following the antidepressant treatment. These differences observed in the two animal species could be interpreted as a species-specific response to stress and to the antidepressant treatment, evidencing that using animal species more similar to human, such as primates, would be very important to study psychiatric disorders. In fact, their brain structures and the mechanisms involved in the regulation of the emotional states have been demonstrated to be more similar to those observed in the human brain; thus, they would be more indicative than rodent species of the human condition. Unfortunately, the poor availability and the ethical issues of using primates in these kinds of studies represent a limit to the improvement of the knowledge of the psychiatric disorders.

5.3 NPY Y_2 RECEPTOR mRNA EXPRESSION IN AMYGDALA AND TEMPORAL CORTEX OF PSYCHIATRIC SUBJECTS

The present study has reported a wealth of interesting pre-clinical results, obtained analyzing the NPY system in the transgenic mice lacking Y_2 receptor gene and in three different animal models of depression. Although one of the animal models studied was in a pre-primate species, depression is a human condition, not reliably reproducible in laboratory animals, thus pre-clinical studies can only provide a simplified model of the depressive-like state. For this reason, to provide a more complete analysis of the involvement of the NPY system in mood disorders, a human post-mortem study involving psychiatric subjects was also considered in the present work. The human tissues used, obtained from the Stanely Foundation Neuropathology Consortium, represent a good tissue collection, in which a high number of subjects composes each group, of great value in view of the difficulties in obtaining human specimen, especially those of psychiatric subjects. In this study, three different pathological groups were considered: major depression, bipolar disorder and schizophrenia, the last group included to have a specificity of the disease. To reduce the variability among the groups, given the high heterogeneity characterizing human subjects, which could affect the final result of the research work, the subjects were matched for a series of factors, such as age, gender, PMI, hemisphere side, use of antidepressant and duration of the treatment, substances of abuse history.

The focus of the present study was the amygdala and the adjacent temporal cortex, brain structures known for their involvement in the regulation of emotions (Sah *et al.,* 2003), emotional memories (Buchanan, 2007) and cognitive functions (Lupien *et al.,* 2007; Watanabe & Sakagami, 2007). The amygdala has been demonstrated to be anatomically related to a number of other brain regions (McDonald, 1998); in particular, cortical inputs supply information from structures related with memory systems (Sah *et al.,* 2003), emotion and vigilance (Davis & Whalen, 2001), which are altered in depression (Drevets *et al.,* 2001). Of the three major NPY receptors in the brain, the present study focused on the Y_2 subtype, which has been demonstrated to be the predominant NPY receptor in the human brain, in which it is abundantly expressed in the amygdala and temporal cortex (Caberlotto *et al.,* 1998a). As demonstrated by many pre-clinical studies, the Y_2 receptors in amygdala are known for their critical role in the regulation of the emotions, stress-related behaviours and cognition and alterations of the expression of the Y_2 receptors in this brain region could be related to the pathophysiology of psychiatric disorders (Nakajima *et al.,* 1997; Sajdyk *et al.,*

2002; Tschenett *et al.,* 2003; Redrobe *et al.,* 2003; Greco & Carli, 2006). However, a previous human post-mortem study has failed in observing a differential Y_2 mRNA expression in the prefrontal cortex of the same psychiatric subjects considered in this study affected by major depression, bipolar disorder and schizophrenia, compared to normal controls (Caberlotto & Hurd, 2001), although a significant effect of suicide as a cause of death has been reported in all these psychiatric groups, in which subjects who have committed suicide have displayed higher Y_2 transcriptional levels in a specific layer of the prefrontal cortex (Caberlotto & Hurd, 2001).

In the present study, the Y_2 receptor mRNA expression in different subnuclei of the amygdaloid complex and in the adjacent temporal cortex of the same groups of subjects considered by Caberlotto and Hurd (2001) was analyzed, but no significant alterations were observed compared to the control subjects. The lack of significant changes of the Y_2 mRNA expression in the amygdala and temporal cortex of subjects affected by depressive disorders seems not to support a role for this NPY receptor in these two brain regions in the regulation of depressive states. Further human post-mortem studies would be needed to demonstrate a possible involvement of this receptor in these two brain regions in the regulation of stress responses and anxiety-related states, rather than depressive disorders, in view of the fact that these regions seem to play a major role in the mechanisms regulating fear, anxiety and cognitive functions (Aggleton, 1993).

In addition, the present study analyzed the existence of a possible relation between the Y_2 receptor mRNA expression and some demographic parameters, showing a positive correlation between the Y_2 transcriptional levels in various amygdaloid nuclei or in the temporal cortical layers and the increasing age of the subjects (Fig. 31). The increased expression of the Y_2 receptor in relation to the age of the subjects seems to be region-specific, since the Y_2 receptor mRNA expression on the human prefrontal cortex was not affected by the age of the patients in the study performed by Caberlotto and Hurd (2001). Moreover, a negative correlation between the Y_2 receptor mRNA expression in the analyzed regions and the post-mortem interval (PMI) was found in the present study (Fig. 31). This finding has been supported by a previous evidence showing the same relation between the Y_2 receptor mRNA expression in the human prefrontal cortex and the PMI (Caberlotto & Hurd, 2001). Altogether, these results underline the importance of a fast recover of human tissues for post-mortem analysis, suggesting caution in selecting brain tissue samples for post-mortem studies, given that the PMI could negatively influence the quality of the tissues used and their ability to yield accurate results (Stan *et al.*, 2006).

Other parameters, such as subjects' gender, use of antidepressants and duration of the treatment, cause of death and hemisphere side analyzed did not affect the Y_2 mRNA expression. In particular, none of the psychiatric groups studied differed from the control subjects on the expression of the Y_2 receptor mRNA in relation to suicide, a cause of death generally highly correlated with mood disorders (Rihmer, 1996), suggesting that the up-regulation of the Y_2 mRNA expression observed in suicide victims in a previous work could be region-specific (Caberlotto & Hurd, 2001). Interestingly, in suicide victims, especially the youngest individuals, a higher Y_1 receptor mRNA expression has been observed in the same brain region (Caberlotto & Hurd, 2001). Moreover, other works have focused on NPY in relation to suicide, also reporting constrasting results: significant differences (Träskman-Bendz *et al.,* 1992) or no alterations (Roy, 1993) have been found on the NPY concentration in the CSF of depressed and non-depressed patients with a recent suicide attempt. Another work considering the NPY-immunoreactivity in post-mortem brain tissues has showed differential NPY concentrations between suicide victims, a subgroup of suicides with a history of depression and subjects dead for natural causes, depending on the brain region considered (Widdowson *et al.,* 1992).

Based on some pre-clinical and clinical recent evidences suggesting a role for NPY and its receptors in drug addiction, especially in the mechanisms related to alcohol intake, dependence and withdrawal (Gilpin *et al.,* 2003; Valdez & Koob, 2004; Thiele *et al.,* 2004; Thorsell, 2007), the effects of the substances of abuse history in relation to the Y_2 mRNA expression were evaluated in this study. Although not much is known about the relation between NPY and cocaine, in this study higher levels of Y_2 mRNA were interestingly found in the accessory basal nucleus of the amygdala in cocaine "habitual users" compared to the "past users", whereas the Y_2 mRNA levels measured in the control subjects resulted intermediate between the other two groups analyzed (Fig. 32). This opposite expression of the Y_2 receptor transcriptional levels between the cocaine "habitual users" and "past users" compared to the control subjects could be due to the possible activation of a mechanism of adaptation associated with the abuse, in which the normal expression of the receptor seemed to be altered. Moreover, the modifed expression of the Y_2 transcriptional levels in the amygdala give further support to the suggested role for this brain region as a key regulator of discrete stimulus-reinforce associations produced by a variety of substances of abuse, ranging from psychostimulants to opiates (See, 2002). In fact, the amygdala has been shown to belong to a cortico-limbic circuit mediating both the acquisition and the expression of conditioning, playing a critical role in relapse to drug-seeking behaviour (See *et al.,* 2003).

Regarding the nicotine use, lower Y_2 transcriptional levels were interestingly observed in the superficial layers of the temporal cortex of the subjects with a history of nicotine abuse compared to the non-users (Fig. 32). A series of recent studies have proposed a role for nicotine in the improvement of cognition and attention and in the attenuation of memory impairment (Levin & Rezvani, 2007; Swan & Lessov-Schlaggar, 2007; Weiss *et al.,* 2007; Potter & Newhouse, 2008; Potter *et al.,* 2006; Xu *et al.,* 2005), suggesting a potential beneficial effect of the nicotinic agonists in the treatment of cognitive and mnemonic dysfunctions (Timmermann *et al.,* 2007; Terry *et al.,* 2005). This result could be related to the known functions of the cerebral cortex in the modulation of cognitive activities, especially in humans (Badre & Wagner, 2007; Briand *et al.,* 2007; Watanabe & Sakagami, 2007) and in particular to the role of NPY in learning and memory processing, known to be exerted especially through the Y_2 receptors (Greco & Carli, 2006; Redrobe *et al.,* 2004). In the present study, a positive correlation between the Y_2 receptor mRNA levels in the temporal cortex and the age of the subjects was found (Fig. 31) and, at the same time, a lower expression of the Y_2 receptors in the cerebral cortex of smokers (Fig. 32), which could be related to a reduction of the age of the cognitive structures in these subjects, possibly due to the beneficial effects of nicotine, which could have anti-ageing effects for the brain functions.

Unexpectedly, no significant results were found in the ethanol abusers, in which the most significant changes on the Y_2 transcriptional levels would have been expected, in view of a number of evidences demonstrating an involvement of the NPY system in alcohol dependence, with a particular focus on the Y_2 receptor antagonists as selective suppressors of the motivation to ethanol self-administration (Thorsell *et al.,* 2006; Badia-Elder *et al.,* 2007; Carvajal *et al.,* 2007; Rimondini *et al.,* 2005; Thiele *et al.,* 2004; Valdez & Koob, 2004) and based on the findings showing a correlation between the ethanol abuse and altered NPY levels in the amygdala, often associated with mood disorders, such as anxiety (Roy & (Pandey *et al.,* 2003a; Pandey *et al.,* 2003b; Roy & Pandey, 2002).

6. CONCLUSION

This study investigated the potential role of NPY and its major receptor subtypes in depression and anxiety using different animal models of depression, transgenic mice lacking one of the most important NPY receptors and post-mortem human specimens of subjects affected by mood disorders. The animal models resulted extremely useful to further analyze the involvement of NPY and particularly of its three major receptor subtypes, Y_1, Y_2 and Y_5, in the mechanisms regulating stress responses and depressive mood. All the receptors were demonstrated to be differentially expressed in the various animal models, showing species-specific changes of their mRNA expression and to be influenced by the exposure to stress. However, it remains to be clarified which receptor subtypes is the most involved in the regulation of mood disorders, even if the present study mainly supported the role of the Y_5 receptor in mood disorders. Moreover, the most frequent and consistent expression changes involving the NPY system were observed in the following brain areas: hippocampal formation, hypothalamus and amygdala; thus, the present findings gave further support for the crucial role of these brain regions in the affective disorders. The role of Y_2 receptor in these kinds of disorders, which was supported by pre-clinical evidences and human post-mortem studies, seemed not to be confirmed in the present study. Further investigations would be necessary to complete the research project started with the present study, in particular using human tissues, given that affective disorders, especially depressive disorders, represent a typical human condition, not perfectly reproducible by animals, which can only provide a simplified model of the depressive-like state. Furthermore, the use of selective receptor antagonists applied to studies involving animal models would provide more detailed and specific results. They would probably indicate the receptor subtype playing the most important role in these disorders and it would be considered the novel target for the discovery and development of new antidepressants and anxiolytic drugs. Unfortunately, this purpose results particularly difficult because of the lack of selective and brain-penetrant antagonists. Hopefully, these compounds will be synthesized in the future, representing a novel and innovative approach in understanding the basis of the mechanisms regulating the NPY role in depression and anxiety and in particular providing a treatment against depression and anxiety disorders.

Finally, an involvement of the Y_2 receptor in drug dependence was observed, in particular in cocaine and nicotine abuse, whereas some previous works have especially

focused on the functions of the NPY system in relation to alcohol intake.

Moreover, the anxiety-related behaviours in two different strains of mice lacking the Y_2 receptor were considered, underlining the importance of the genetic background in influencing the anxiety-like states, showing that the different genetic component could have a stronger impact on the phenotype than the deletion of an entire gene and suggesting caution in interpreting the data obtained from studies on knockout mice.

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