Chronic psychosocial stress alters NPY system: Different effects in rat and tree shrew

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A B S T R A C T

The neuropeptide Y (NPY) system has been largely studied in relation to affective disorders, in particular for its role in the mechanisms regulating the pathophysiology of anxiety and depression and in the stress-related behaviours. Although NPY has been previously investigated in a variety of animal models of mood disorders, the receptor subtype mainly involved in the modulation of the stress response has not been identified. In the present study, the chronic psychosocial stress based on the resident–intruder protocol—an ethologically relevant paradigm known to induce behavioural and endocrine modifications which mimic depression-like symptoms—was used. Two different species were investigated: rat and tree shrew (Tupaia belangeri); the latter is regarded as an intermediate between insectivores and primates and it was chosen in this study for its pronounced territoriality. In these animals, the regulation of NPY and of Y1, Y2 and Y5 receptors mRNA expression was evaluated after chronic stress and chronic antidepressant treatment by in situ hybridization in selected brain regions known to be involved in the pathophysiology of mood disorders. The animals were exposed to psychosocial stress for 35 days and concomitant daily fluoxetine treatment (10 mg/kg for rats and 15 mg/kg for tree shrews) after the first week of stress. The results confirmed a major role for hippocampal and hypothalamic NPY system in the pathophysiology of mood disorders. Although there were no evident differences between rat and tree shrew in the NPY system distribution, an opposite effect of chronic psychosocial stress was observed in the two species. Moreover, chronic antidepressant treatment was able to counteract the effects of stress and restored basal expression levels, suggesting the utility of these paradigms as preclinical models of stress-induced depression. Overall, although evident species differences were found in response to chronic psychosocial stress, the present study suggests a role for NPY receptors in the stress response and in the action of antidepressant drugs, providing further support for an involvement of this neuropeptidergic system in the pathophysiology of depression and anxiety.

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1. Introduction

The study of the mechanisms involved in the etiology of affective disorders requires the investigation of animal models potentially reflecting the course and symptoms of human pathologies. In addition to genetic factors, which are known to predispose to psychopathologies (McGuffin and Katz, 1989), environmental stress plays an important role in the etiology of anxiety and depression, which are linked to maladaptive changes in the stress response (Kendler et al., 1995a,b).

Abbreviations: ACTH, adrenocorticotropin hormone; ANOVA, analysis of variance; CA1, CA1 region of hippocampus; CA2, CA2 region of hippocampus; CA3, CA3 region of hippocampus; CNS, central nervous system; DG, dentate gyrus; HPA, hypothalamic-pituitary-adrenocortical; NPY, Neuropeptide Y; PBS, phosphate buffered saline; SSC, saline sodium citrate; VMH, ventro-medial hypothalamus; VMHDM, ventro-medial hypothalamus, dorso-medial portion.

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Since the majority of stressful stimuli leading to psychopathologies in humans are of social nature (Bjorkqvist, 2001; Kendler et al., 2003; Kessler, 1997; Kessler et al., 1985), the study of the consequences of social stress in experimental animal models is of great interest. Social defeat using the resident–intruder paradigm (Tornatzky and Miczek, 1994) represents a suitable and naturalistic experimental method to study the effects of stress (Fuchs et al., 1996). Subordinate animals exhibit physiological and behavioural changes similar to those of depressive-like state, such as increased adrenocorticotropic hormone (ACTH) and glucocorticoid activity (Buwalda et al., 1999, 2001), disturbances in sleep (Rüther, 1989), altered heart rate, blood pressure and core temperature (Meerlo et al., 1996; Spínolo et al., 1999), impaired immunological function and reduced resistance to diseases (Engler et al., 2004; Stefanski and Engler, 1998), decreased locomotor and exploratory activities (Koolhaas et al., 1997; Meerlo et al., 1996; Rygula et al., 2005), reduced self-grooming (van Erp et al., 1994), impaired consummatory behaviour, with a consequent loss of body weight (Kramer et al., 1999; Rybkin et al., 1997), reduced aggression and sexual behaviour.
Neuropeptide Y (NPY) is a neuroactive peptide acting as a neurotransmitter and neuromodulator regulating many physiological functions (Colmers and Wahlestedt, 1993). Preclinical and clinical studies support the role of NPY in the regulation of emotions and stress-related behaviours, showing that increased NPY levels in specific regions of the central nervous system (CNS) induce antidepressant- and anxiolytic-like effects, whereas a down-regulation of this peptide induces opposite effects on the emotional responses (Heilig, 2004). Since the NPY system is altered by stressful challenges (Castagné et al., 1987), the study of its effects on the emotional responses (Heilig, 2004). Since the NPY system 2002; Jiménez-Vasquez et al., 2001) have demonstrated alterations of 2006) or environmental models (Husum and Mathé, 2002; Husum et al., 2008, 2001; Jiménez-Vasquez et al., 2000a,b; Wortwein et al., 2007), while the Y1 antagonists induce anxiety and depressive-like behaviour in various animal models of anxiety (Primeaux et al., 2005; Redrobe et al., 2002; Sajdyk et al., 1999). Conversely, pharmacological or genetic blockade of Y2 receptors has been shown to induce anxiolytic- and antidepressant-like profiles (Bacchi et al., 2006; Carvajal et al., 2006; Redrobe et al., 2003; Tschennet et al., 2003). Among the other NPY receptors, the Y3 receptor subtype has been mainly studied in relation to food intake (Schaffhauser et al., 1997; Kask et al., 2001), however, based on its distribution in brain regions known for their role in emotional disorders (Dumont et al., 1998; Gerald et al., 1996), Y3 has been suggested to be involved in the regulation of anxiety-like state and in the response to stressful stimuli (Sajdyk et al., 2002; Sørensen et al., 2004). The remaining receptor subtypes were not considered in this study; the Y4 receptor subtype has not yet been cloned (Lee and Miller, 1998), while the Y5 receptor was primarily found in peripheral tissues (Barrios et al., 1999). The Y6 receptor gene was found to be completely absent in the rat (Burkhoff et al., 1998) and to be functional only in some species but not in human and other primates (Gregor et al., 1996; Matsumoto et al., 1996), whereas the Y7 receptor has been cloned only in fish, amphibians and chicken (Fredriksson et al., 2004; Bromée et al., 2006).

Therefore, the present study was focused on the characterization of the three major NPY receptors—Y1, Y2 and Y5—in order to understand their involvement in the regulation of the NPY role in the emotional states. This was investigated using a validated stress-induced animal model of depression, the chronic psychosocial stress, an ethological stress paradigm inducing depressive-like symptoms (Fuchs et al., 1996, 2001; Kramer et al., 1999) and evaluating the effects of the established antidepressant fluoxetine to counteract the alterations induced by stress. Two different animal species were investigated, a rodent (rat) and a pre-primate (tree shrew), the latter phylogenetically classified as an intermediate between insectivores and primates (Martin, 1990). In addition, since contrasting evidence about the receptor subtype mainly involved in the mediation of the role of NPY in the regulation of emotions still exists, the potential changes of the different receptor subtypes were also considered, in order to identify the receptor subtype primarily involved in the regulation of emotional processes.

2. Materials and methods

2.1. Animal care and treatments

Tissues from adult male rats and tree shrews were obtained from cohorts submitted to social conflict paradigm at the German Primate Center (Göttingen, Germany).

The experimental design of the study, the behavioural and hormonal characterisations of the rats used in the present study were previously described in detail (Rygula et al., 2006; Fig. 1). The rats used in the present study are the same as those included in the work of Rygula and colleagues. Three different experimental phases and four groups of animals (n = 6 rats per group) were generated: i) Control, ii) Stress, iii) Control + fluoxetine, iv) Stress + fluoxetine. The first experimental phase lasted for 7 days, during which all the animals were handled daily and body weight was recorded. The second phase also lasted for 7 days, during which the Wistar rats (intruders) of the Stress+ groups of animals (Rygula et al., 2006) and Stress+fluoxetine groups were exposed daily to 1 h of psychosocial stress. The third experimental phase, lasting 28 days, consisted of the antidepressant treatment: stressed rats were maintained in the psychosocial conflict situation and were treated daily with fluoxetine or vehicle. Animals in the Control + fluoxetine and Stress + fluoxetine groups received fluoxetine (10 mg/kg body weight per day) orally directly before or after the defeat sessions. The drug (Fluoxetine Ratiopharm®, 4 mg/ml oral solution; Ratiopharm GmbH, Ulm, Germany) was administered using a bulb-headed cannula into the buccal cavity to minimize uncontrollable stress effects caused by daily injections. This dose has been demonstrated to be effective in reversing a series of endocrine and behavioural parameters modified by stress exposure (Rygula et al., 2006) and resulting with blood concentration of fluoxetine and its major active metabolite, norfluoxetine, similar to those reported in human patients treated with therapeutically active doses (Czéh et al., 2006). The animals of the Control and Stress groups were treated with vehicle only. On the last experimental day (day 42), all the animals were sacrificed, the brains were rapidly

![Fig. 1. Experimental groups and design of the study.](image-url)
removed, immediately frozen over liquid nitrogen, stored at −80 °C and sent to GlaxoSmithKline laboratories for in vitro assays.

The experimental design of the study on the tree shrews has been previously reported (Czéh et al., 2006). The experimental protocol was similar to that used in the rat model, with the difference that the animals were 4 per group and the daily administered dose of fluoxetine was 15 mg/kg. This dose has been demonstrated to be effective in restoring the hormonal, behavioural and morphological alterations induced by the exposure to stress in a previous study and resulting with serum concentration of fluoxetine and its major active metabolite close to the range reported for patients under fluoxetine treatment (Czéh et al., 2006). The behavioural and endocrine characterisation of these animals after exposure to stress corresponded to that described by Fuchs (2005) and fluoxetine was able to counteract the effects of stress.

Animal experiments were conducted according to the European Council Directive of November 24, 1986 (86/609/EEC), and were approved by the Lower Saxony Federal State Office for Consumer Protection and Food Safety, Germany. The minimum number of animals required to obtain consistent data was used.

2.2. Tissue samples
Concoral 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, 1998). The corresponding levels in the tree shrew brain (Tigges and Shantha, 1969) were also cut. The slices were thaw-mounted onto polarized SuperFrost Plus slides and stored at −80 °C until usage.

2.3. Riboprobes preparation

The riboprobes corresponding to rat NPY, Y1 and Y2 receptors mRNA were generated as previously described (Zambello et al., 2008). The rat Y5 receptor riboprobe was made from a 600 bp cDNA of the entire Y5 mRNA sequence (NM_012869), spanning from the amino acids 703–1302 of the receptor and then subcloned in a pCRII-TOPO vector (invitrogen). The probe specificities were evaluated with the public domain program Basic Local Alignment Search Tool (BLAST) in the NCBI package. No sequences displayed significant similarity with other sequences in the Non-Redundant database at NCBI. Rat riboprobes were also used for hybridizing tree shrew brain sections, given the known radioactivity of the14C standards relative to their translocation levels, the light transmittance values per mm2 [PLS/mm2] were determined with a computerized image analysis system (AIS 4.0, Imaging Research, St. Catharines, Ontario, Canada). Based on the known radioactivity of the 14C standards relative to their translocation levels, the light transmittance values per mm2 were converted to ng/g using a calibration curve. All the regions of interest belonged to the forebrain—i.e. cingulate cortex, septum, CA regions and dentate gyrus (DG) of hippocampus, amygdaloid and hypothalamic nuclei—and were chosen among those regions which reported changes in depression and stress-related disorders. They were defined by anatomical landmarks in conjunction with a rat brain atlas (Paxinos and Watson, 1998) and a tree shrew brain atlas (Tigges and Shantha, 1969).

Generally, two consecutive sections were considered for each subject, i.e. cingulate cortex, septum, CA regions and dentate gyrus (DG) of hippocampus, amygdaloid and hypothalamic nuclei—and were chosen among those regions which reported changes in depression and stress-related disorders. They were defined by anatomical landmarks in conjunction with a rat brain atlas (Paxinos and Watson, 1998) and a tree shrew brain atlas (Tigges and Shantha, 1969). Generally, two consecutive sections were considered for each subject, based on the anatomy, and the regions were bilaterally analyzed and averaged. The measurements of each specific brain region were taken by individually tracing the structures on the TV monitor with a cursor.

2.4. In situ hybridization

Tissue sections were warmed to room temperature and allowed to dry, then they were fixed in 4% formaldehyde/1× phosphate buffered saline (PBS) for 5 min, rinsed twice in 1× 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 min. The sections were then rinsed in 2× saline sodium citrate (SSC), dehydrated in a graded series of ethanol (70%, 80%, 95%, 100%) and delipidated with chloroform. They were then 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, 2× Denhardt’s solution, 20% dextran sulfate, 8× SSC and 50% formamide. Before the hybridization, the labelled probe was added to the hybridization mixture in the concentration of 2× 106 cpm/µl and 150 µl of this hybridization solution was applied to each slide. The sections of tissue were coveredslipped to prevent evaporation and hybridization was carried out in a humidified chamber overnight at 55 °C. Incubation was followed by washes in a graded series of SSC (2×, 1×, 0.5×, 0.1×), all at room temperature except for 0.1× 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-TR2025) together with 14C standards for 4–5 days.

2.5. 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 14C standards relative to their translocation levels, the light transmittance values per mm2 were converted to ng/g using a calibration curve. All the regions of interest belonged to the forebrain—i.e. cingulate cortex, septum, CA regions and dentate gyrus (DG) of hippocampus, amygdaloid and hypothalamic nuclei—and were chosen among those regions which reported changes in depression and stress-related disorders. They were defined by anatomical landmarks in conjunction with a rat brain atlas (Paxinos and Watson, 1998) and a tree shrew brain atlas (Tigges and Shantha, 1969).

Generally, two consecutive sections were considered for each subject, based on the anatomy, and the regions were bilaterally analyzed and averaged. The measurements of each specific brain region were taken by individually tracing the structures on the TV monitor with a cursor.

2.6. Statistical analysis

Statistical evaluations of the differences among the experimental groups were assessed using “Statistica 6.0” software package. Data were analyzed by three-way ANOVA (analysis of variance), considering the pharmacological treatment (fluoxetine/vehicle), the stress exposure (stress/no stress) and the brain regions as factors and NPY or receptor mRNA expression as dependent variable. The ANOVA was followed by planned comparisons and Newman–Keuls post-hoc test. All the results were expressed as mean ± S.E.M. and the p-values were considered significant if lower than 0.05.

<table>
<thead>
<tr>
<th>Riboprobe</th>
<th>cDNA fragment length (bp)</th>
<th>Plasmid vector</th>
<th>Restriction enzyme generating sense riboprobe</th>
<th>Restriction enzyme generating antisense riboprobe</th>
</tr>
</thead>
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<tr>
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<td>pGEMZ4</td>
<td>Dral/T7</td>
<td>PstI/S6</td>
</tr>
<tr>
<td>Rat Y1</td>
<td>245 bp</td>
<td>Bluescript II SK</td>
<td>PstI/T7</td>
<td>EcoRI/T3</td>
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<td>Rat Y2</td>
<td>423 bp</td>
<td>pBSKII</td>
<td>BamHI/T3</td>
<td>XhoI/T7</td>
</tr>
<tr>
<td>Rat Y5</td>
<td>600 bp</td>
<td>pCRII-TOPO</td>
<td>XhoI/S6</td>
<td>HindIII/T7</td>
</tr>
</tbody>
</table>
3. Results

3.1. NPY mRNA expression in the rat brain

The statistical analysis of NPY mRNA expression levels evaluated in the four experimental groups of rats revealed a significant difference in the NPY mRNA expression levels in the CA3 hippocampal region between Control and Stress group (p = 0.011), with lower levels in the stressed animals. In the DG of hippocampus, a significant effect of the interaction between exposure to chronic psychosocial stress and fluoxetine treatment was detected (p = 0.018), with a down-regulation in the Stress group of rats compared to the Control rats and an up-regulation in the Stress + fluoxetine group compared to the Control + fluoxetine group (Fig. 2A).

In all the other regions analyzed, cingulate cortex, septum, medial amygdala, CA1 and CA2 hippocampal regions and arcuate nucleus of hypothalamus, no significant changes were detected.

3.2. Y1 receptor mRNA expression in the rat brain

A significant stress × treatment interaction was found in the Y1 receptor mRNA expression levels in the ventro-medial hypothalamus (VMH; p = 0.037), in which a down-regulation was measured in the Stress group of rats compared to Control animals and an up-regulation was observed in the Stress + fluoxetine animals compared to the Control + fluoxetine group (Fig. 2B).

No significant differences were found in all the other regions considered (cingulate cortex, septum, central, medial and basolateral amygdala, paraventricular, and arcuate hypothalamic nuclei, CA1, CA2, CA3 and DG of hippocampus).

3.3. Y2 receptor mRNA expression in the rat brain

A significant difference in Y2 receptor mRNA expression levels was observed between Control and Stress rats in the ventro-medial hypothalamus, dorso-medial portion (VMHDM; p = 0.027), in which lower Y2 receptor mRNA levels were found in the stressed rats compared to control rats (Fig. 2C).

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 DG of hippocampus.

3.4. Y5 receptor mRNA expression in the rat brain

A significant effect of the interaction between exposure to psychosocial stress and fluoxetine treatment was observed in the VMH.

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Fig. 2. NPY system mRNA levels detected in the brains of rats exposed to chronic psychosocial stress protocol. (A) NPY mRNA expression levels in the CA3 region and in the dentate gyrus (DG) of hippocampus of rats belonging to the Control, Control + fluoxetine, Stress and Stress + fluoxetine groups. 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). In the CA3 region: * p < 0.05: effect of stress. In the DG: * p < 0.05: effect of stress × treatment interaction. (B) Y1 receptor mRNA expression levels in the ventro-medial hypothalamus (VMH) of rats belonging to the Control, Control + fluoxetine, Stress and Stress + fluoxetine groups. The bar graph represents the mean ± S.E.M. (n = 6 rats/group) given as nCi/g (VMH = 463.5 ± 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: effect of stress × treatment interaction. (C) Y2 receptor mRNA expression levels in the ventro-medial hypothalamus, dorso-medial portion (VMHDM) of rats belonging to the Control, Control + fluoxetine, Stress and Stress + fluoxetine groups. 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: effect of stress. (D) Y5 receptor mRNA expression levels in the ventro-medial hypothalamus (VMH) of rats belonging to the Control, Control + fluoxetine, Stress and Stress + fluoxetine groups. 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 ± 1.36 in the Stress rats and VMH = 23.5 ± 1.62 in the Stress + fluoxetine rats). * p < 0.05: effect of stress × treatment interaction.
(p = 0.041), in which a significant down-regulation of Y5 receptor mRNA expression levels was measured in the rats of the Stress group compared to the Control rats, while an up-regulation was found in the Stress + fluoxetine group compared to the Control + fluoxetine (Fig. 2D).

All the other regions analyzed (cingulate cortex, septum, central, medial and basolateral amygdala, paraventricular and arcuate hypothalamic nuclei, hippocampal CA1, CA2, CA3 regions and DG) did not reveal significant variations of Y5 receptor mRNA expression.

3.5. NPY mRNA expression in the tree shrew brain

The distribution of NPY and Y1, Y2, Y5 receptor mRNA obtained in the tree shrew brain using riboprobes complementary to rat mRNA sequences was very similar to the distribution of the NPY system in the rat brain (Fig. 3).

The statistical analysis of the NPY mRNA expression levels evaluated in the four experimental groups of animals showed a significant difference in the NPY mRNA levels between Control and Stress animals, with higher NPY mRNA expression levels in the CA1 (p = 0.007) and CA2 (p = 0.034) hippocampal regions and in the arcuate nucleus of the hypothalamus (p = 0.01) after exposure to psychosocial stress (Fig. 4A). In addition, the post-hoc analysis (Newman–Keuls test) showed a trend in the CA1 hippocampal region between Control and Stress groups (p = 0.056), between Control and Stress + fluoxetine groups (p = 0.092) and between Control + fluoxetine and Stress groups (p = 0.083). The same analysis conducted for the CA2 hippocampal region evidenced a trend between Control and Stress groups (p = 0.068), between Control + fluoxetine and Stress groups (p = 0.051) and between Stress and Stress + fluoxetine groups (p = 0.058). Additionally, in the arcuate hypothalamic nucleus, the post-hoc analysis evidenced a significant interaction between Control and Stress groups (p = 0.047) and between Control + fluoxetine and Stress groups (p = 0.027). It is of great interest to mention that in all these regions, in particular in the arcuate hypothalamic nucleus, fluoxetine counteracted the effect of the chronic psychosocial stress by inducing a reduction of the NPY mRNA levels in the Stress + fluoxetine group and restoring the initial conditions.

No significant differences were found in all the other regions analyzed, such as cingulate cortex, septum, medial and basolateral amygdala, CA3 region and DG of hippocampus.

3.6. Y1 receptor mRNA expression in the tree shrew brain

The statistical analysis did not evidence any alteration in the tree shrew Y1 receptor mRNA expression levels after exposure to stress, or induced by 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 DG of hippocampus.

3.7. Y5 receptor mRNA expression in the tree shrew brain

The Y5 receptor mRNA expression reported significant differences between Stress and Control groups in the CA3 hippocampal region, with higher levels in the stressed rats compared to control (p = 0.043; Fig. 4B).

In all the other regions considered, such as cingulate cortex, septum, medial and basolateral amygdala, CA1, CA2, CA3 regions and DG of hippocampus no significant changes were observed.

3.8. Y3 receptor mRNA expression in the tree shrew brain

The statistical analysis of the Y3 receptor mRNA expression in the tree shrew brain reported a significant effect of the treatment with fluoxetine in the cingulate cortex, in which lower Y3 mRNA levels were observed in the fluoxetine treated groups of animals compared to non-treated animals (p = 0.028; Fig. 4C). In addition, in the same region a stress × treatment interaction was found, with an up-regulation measured in the Stress group of tree shrews compared to Control animals and a down-regulation in the Stress + fluoxetine group compared to Control + fluoxetine animals (p = 0.039; Fig. 4C). Moreover, in the same region, the post-hoc analysis (Newman–Keuls test) evidenced a significant interaction in the Y5 mRNA expression between Stress and Stress + fluoxetine groups (p = 0.027), while a trend (p = 0.064) was found between Control and Stress groups. Furthermore, a difference due to the interaction between stress and fluoxetine was observed in the CA1 (p = 0.044) and CA2 (p = 0.031) hippocampal regions, in which lower Y5 mRNA levels were measured in the Stress group compared to Control and higher levels were found in the Stress + fluoxetine compared to Control + fluoxetine group (Fig. 4C).

No significant differences were measured in all the other regions analyzed, such as septum, medial and basolateral amygdala, CA3 and DG of hippocampus.

4. Discussion

The impact of stressful events on the development of pathologies of the brain has been widely investigated and the use of social conflict between members of the same species is a natural stressor leading to a variety of long-lasting physiological, behavioural and molecular changes (Björkqvist, 2001; Fuchs and 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 discrete brain areas (Fuchs and Flugge, 2002; Krugers et al., 1993; Isovich et al., 2001; McKittrick et al., 2000). Moreover, a number of studies have reported that these changes were reversed by treatment with antidepressant drugs (Fuchs et al., 1996, 2004; Kramer et al., 1999; Rygula et al., 2006), confirming that chronic psychosocial stress represents a validated animal model of depression. Based on these premises, the present study investigated potential transcriptional changes of the NPY system in two different animal species, a rodent and a pre-primate, chronically exposed to psychosocial stress and the possible effect of fluoxetine, an established antidepressant widely used in clinical protocols. The purpose of this study was to assess if the mechanism by which chronic psychosocial stress induces a depressive-like state is mediated by this neuropeptidergic system and if this system is involved in the mechanism of action of an antidepressant drug.

Considering the rat, the NPY mRNA levels were found to be significantly decreased in the DG and CA3 hippocampal region of the stressed animals compared to controls (Fig. 2A), in line with previous findings involving a variety of animal models of depression, both genetic and stress-induced (Bjørnebekk et al., 2006; Caberlotto et al., 1998; Humus and Mathé, 2002; Jiménez-Vasquez et al., 2007; Mathé et al., 1998) and in support of the hypothesis that reduced hippocampal NPY levels are strictly related to the depressive-like state of the animal (Heilig, 2004). In particular, in the DG of hippocampus, an opposite regulation between stress exposure and antidepressant treatment was observed, consisting of a down-regulation of NPY mRNA expression in the stressed rats, reversed by chronic administration of fluoxetine, which restores control NPY levels (Fig. 2A). This result gives further support for a role of the hippocampal NPY system in the regulation of the neurobiological circuitry of emotional processes (Heilig, 2004). The present findings showing reduced NPY mRNA expression in the hippocampal CA3 region and DG of stressed rats could be partly related to the functional impairment and loss of hippocampal GABAergic interneurons, which are NPY positive (Köhler et al., 1986; Deller and Leranth, 1990), observed under extreme, prolonged stress exposure (McEwen and Magarinos, 1997).

The present study gave further support to the existence of an anatomical and functional link between hippocampus and hypothalamus. As previously described, NPY mRNA was modulated only in the
hippocampus, whereas all the changes affecting NPY receptors were observed in hypothalamic nuclei. In particular, Y₁ and Y₅ receptors displayed a reciprocal regulation between stress exposure and fluoxetine treatment in the ventro-medial-hypothalamus (VHM), with reduced transcriptional levels in the stressed rats compared to controls and restored mRNA levels following fluoxetine treatment (Fig. 2B,D).
Fig. 4. NPY system mRNA levels detected in the brains of tree shrews exposed to chronic psychosocial stress protocol. (A) NPY mRNA expression levels in the CA1 and CA2 hippocampal regions (on the left) and in the arcuate nucleus (Arc) of hypothalamus (on the right) of tree shrews belonging to the Control, Control + fluoxetine, Stress and Stress + fluoxetine groups. The bar graph represents the mean ± S.E.M. (n = 4 animals/group) given as nCi/g (CA1 = 77.85 ± 5.3 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 Stress + 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). In the CA1 region: "p < 0.01: effect of stress. In the CA2 region: * p < 0.05: effect of stress. In the Arc: "p < 0.01: effect of stress; Newman–Keuls post-hoc test: * p < 0.05 compared to Stress group. (B) Y2 receptor mRNA expression levels in the CA3 hippocampal region of tree shrews belonging to the Control, Control + fluoxetine, Stress and Stress + fluoxetine groups. 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: effect of stress. (C) Y5 receptor mRNA expression levels in the cingulate cortex (CC, on the left), CA1 and CA2 hippocampal regions (on the right) of tree shrews belonging to the Control, Control + fluoxetine, Stress and Stress + fluoxetine groups. 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 = 12.64 ± 0.29 in the Stress group; CC = 11.12 ± 0.31 in the Stress + fluoxetine group; CA3 = 23.54 ± 0.76 in the Control group; CA3 = 20.56 ± 1.34 in the Control + fluoxetine animals; CA1 = 19.32 ± 2.34 in the Stress group; CA1 = 23.56 ± 1.3 in the animals of the Stress + fluoxetine group; CA2 = 32.06 ± 1.13 in the Control group; CA2 = 28.03 ± 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). In the CC: * p < 0.05: effect of stress and stress x treatment interaction; Newman–Keuls post-hoc test: + p < 0.05 compared to Stress group. In the CA1 and CA2 regions: "p < 0.05: effect of stress × treatment interaction.

These changes in Y1 and Y5 receptors were consistent with the hypothesis of their anatomical and functional interactions. They have been demonstrated to co-localize in several rat brain regions (Wolak et al., 2003) and a high dimerization state of these two receptor subtypes has been observed (Dinger et al., 2003), suggesting that they potentially exert a reciprocal regulation (Gehlert, 2004; Gehlert et al., 2007; Lin, 2004). Moreover, unlike the other NPY receptor subtypes, Y1 and Y5 functions in stress-related responses and depressive-like states.

A significant reduction of the Y2 receptor transcriptional levels was observed in the ventro-medial hypothalamic nucleus, dorso-medial portion (VMHDM) of the stressed rats, compared to non-stressed (Fig. 2C), suggesting that the chronic psychosocial conflict could be linked to alterations of the hypothalamic-pituitary adrenocortical (HPA) axis (Fuchs et al., 2001; Kramer et al., 1999). Consistent with this hypothesis, a parallel investigation of physiological parameters measured in these rats showed that the exposure to chronic psychosocial stress induces an increase in adrenal weight (Rygula et al., 2006), indicative of the effects of stress (Sapolsky et al., 2000), psychosocial stress induces an increase in adrenal 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 and Willner, 1992). The increased adrenal weight observed in the stressed animals might reflect a hyper-activation of the HPA axis (Kramer et al., 1999; Fuchs et al., 2001), a dysregulation representing one of the best replicated findings in human depressed patients (Rubin et al., 1987), further confirming the validity of the model.

Although animal models of depression are prevalently focused on rodents, in order to better investigate human condition, the present
study considered the effects of the chronic psychosocial stress on the NPY system in a non-rodent species, the tree shrew (*Tupaia belangeri*). Tree shrews are phylogenetically regarded as an intermediate between insectivores and primates and show a high genetic homology with humans (Martin, 1990), a pronounced territoriality in male animals and a day-active life (Fuchs et al., 1996). Based on these characteristics, the effect of chronic psychosocial stress on tree shrews could represent another 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 show behaviours mirroring symptoms of human depressed patients and respond to chronic antidepressant treatments, leading to an improvement of the symptoms (Fuchs, 2005; Fuchs and Flugge, 2002; Fuchs et al., 1996).

In contrast to the results in rats, chronic exposure to psychosocial stress in the tree shrews induced an increase of NPY mRNA levels in the CA1-2 hippocampal regions and in the arcuate hypothalamic nucleus (Fig. 4A). Although it was not easy to find an explanation to clarify the contrasting results in the two species, the observed up-regulation of NPY mRNA in the tree shrew could be due to a response of the NPY system to the stress-induced reduction of hippocampal neurogenesis in the DG observed in chronically stressed animals in parallel studies (Czéh et al., 2006, 2001; Fuchs et al., 2001). Indeed, the transcriptional up-regulation, particularly in the hippocampal formation, could be explained by the hypothesis of a neuroprotective role of NPY, which counteracts the effect of stress in the hippocampal cells. Indeed, NPY has been shown to contribute to the maintenance of the hippocampal neurogenesis, thus suggesting a possible neuroprotective function against stress-induced cellular damage in the hippocampus (Howell et al., 2003, 2005, 2007). It could be speculated that NPY exerts its antidepressant properties and its improvement of learning and memory processing partly mediating its activity on hippocampal neuroproliferation.

The Y5 receptor mRNA expression was increased in the CA3 hippocampal region of the stressed tree shrews compared to the non-stressed animals (Fig. 4B), confirming the suggested relevant role of Y5 receptor in the regulation of emotional behaviour, supported by an increase of Y5 mRNA in human post-mortem brains of suicides (Cabrelotto and Hurd, 2001).

The NPY Y5 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 of stressed animals, recovering the levels of the non-stressed animals after treatment with fluoxetine (Fig. 4C), as previously described for rats. Stress exposure also increased Y5 receptor mRNA levels in other limbic regions, such as medial amygdala and cingulate cortex. The Y5 receptor has been prevalently studied for its involvement in the mechanisms related to food intake and obesity (Gehlert, 1999; Cabrele et al., 2000). The results presented here are particularly interesting, since they represent the first example of Y5 mRNA alteration in an animal model of depression and provide evidence for its involvement in the mechanism regulating the response to stress.

In conclusion, this study underlines that the NPY system transcriptional levels are affected by chronic psychosocial stress both in rat and tree shrew and demonstrated the ability of fluoxetine in preventing the effect of stress, by restoring the NPY system mRNA to control levels. In addition, these findings demonstrated that the three major NPY receptors, Y1, Y2 and Y5 could have a role in modulating the NPY response to stress and this could be considered for a potential therapeutic treatment.

Finally, the results of this work have highlighted the existence of species-specific molecular mechanisms of response to stress and to the antidepressant treatment, which suggest the need of evaluating behavioural models in different animal species for a better understanding of psychiatric disorders.

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Caberlotto L, Jimenez P, Hurd YL, Mathé AA, Fuxe K. Alterations of the NPY system to the stress-induced reduction of hippocampal neurogenesis in the DG observed in chronically stressed animals in parallel studies (Czéh et al., 2006, 2001; Fuchs et al., 2001). Indeed, the transcriptional up-regulation, particularly in the hippocampal formation, could be explained by the hypothesis of a neuroprotective role of NPY, which counteracts the effect of stress in the hippocampal cells. Indeed, NPY has been shown to contribute to the maintenance of the hippocampal neurogenesis, thus suggesting a possible neuroprotective function against stress-induced cellular damage in the hippocampus (Howell et al., 2003, 2005, 2007). It could be speculated that NPY exerts its antidepressant properties and its improvement of learning and memory processing partly mediating its activity on hippocampal neuroproliferation.

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