Neuropeptide Y (NPY) as a modulator of neuroplasticity and emotional behavior in animal models

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Abstract

Neuropeptide Y (NPY) is considered an important neuromodulator in the regulation of emotional behavior. NPY consistently modulates anxiety-related behaviors and there is increasing support for a role for this peptide in mood disorders such as depression. There is also evidence that NPY promotes neuroplasticity in the central nervous system. Recent studies suggest that chronic depression is associated with neuronal loss and abnormalities in neuroplasticity (such as neurogenesis) in the hippocampus. Consequently, the aim of this study was to elucidate the role of the NPY Y_1 and Y_2 receptor subtypes in anxiety- and depression-like behaviors using NPY knockout and transgenic animals and to investigate the role of NPY as a neuroproliferative factor in hippocampal neurogenesis. The ability of NPY to induce changes in neurogenesis was assessed in NPY Y_2 knockout mice and in Sprague-Dawley rats after chronic treatment with NPY.

Résumé

Le Neuropeptide Y (NPY) est considéré comme étant un neuromodulateur important des émotions. Le NPY serait impliqué dans la modulation des comportements anxieux et dans les troubles de l'humeur tels que la dépression. Il y a aussi des évidences qui suggèrent que le NPY favorise la neuroplasticité dans le système nerveux central. Des études récentes out montré que la dépression chronique est associée à une perte neuronale et à des problèmes lors de la neurogenèse dans l' hippocampe. En conséquence, l'objectif de notre étude était d'élucider le rôle des récepteurs Y_1 et Y_2 du NPY dans l'anxiété et la dépression en utilisant des animaux transgéniques ou n'ayant plus le gène de certains récepteur du NPY. Le rôle du NPY comme facteur de la neuroprolifération a aussi été étudié dans la neurogenèse de l'hippocampe. Les changements induits par le NPY lors de la neurogenèse ont été évaluér chez des souris dont le gène du récepteur NPY Y_2 a été supprimé et chez des rats Sprague-Dawley après un traitement chronique avec le peptide NPY.

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Abbreviations

3,3′-diaminobenzidine	DAB
Basolateral nucleus of the amygdala	BLA
Bromodeoxyuridine	BrdU
Central nervous system	CNS
Central nucleus of the amygdala	CeA
Cerebrospinal fluid	CSF
Chronic mild stress	CMS
Corticotropin releasing factor	CRF
Dentate gyrus	DG
Electroconvulsive therapy	ECT
Flinders Resistant Line	FRL
Flinders Sensitive Line	FSL
Granule cell layer	GCL
Hilus	H
Intracerebroventricular	ICV
Knockout	$^{-/-}$
Medial amygdala	MeA
Monoamine oxidase inhibitors	MAOI
Neuropeptide Y	NPY
Neuropeptide Y-like immunoreactivity	NPY-IR
Noradrenaline reuptake inhibitors	NARI
Noradrenergic and specific serotonergic antidepressants	NASSA
Olfactory bulbectomy	OBX
Pancreatic polypeptides	PP
para-chloroamphetamine	PCA
Peptide YY	PYY
Selective serotonin reuptake enhancer	SSRE
Selective serotonin reuptake inhibitors	SSRI
Subgranular zone	SGZ
Subventricular Zone	SVZ
Tricyclic/Tetracyclic antidepressants	TCA
Wildtype	$^{+/+}$

Chapter 1: Introduction

1.1 Preface

Neuropeptide Y (NPY) was isolated from porcine brain more than two decades ago [1]. This 36 amino acid residue is one of the most abundant peptides found in the central nervous system (CNS) of all mammals, including humans [2-4]. It is one of the most conserved peptides in evolution [5;6] suggesting an important role in the regulation of basic physiological functions [7]. At present, five NPY receptor subtypes have been cloned and designated Y_1, Y_2, Y_4, Y_5 and y_6 [8-10]; all of which couple to G_i proteins and inhibit the production of cyclic AMP [11]. NPY has important modulatory functions in the immune and cardiovascular systems [12;13], circadian rhythms [14], food intake [15], and seizure [16]. NPY is consistently involved in anxiety-related behaviors [17] and there is increasing support for the role of NPY in mood disorders such as depression [18].

There is evidence that an alteration of neuroplasticity, specifically hippocampal neurogenesis, may be responsible for the etiology of mood disorders like depression [19]. Neuroplasticity is a lifelong process that mediates the structural and functional reaction of dendrites, axons, and synapses to experience, attrition and injury [20]. The manifestations of neuroplasticity in the adult CNS include but are not limited to: alterations of dendritic ramifications, synaptic remodeling, axonal sprouting, neurite extension, synaptogenesis, and neurogenesis [21]. NPY was recently implicated as an inducer of neuronal precursor proliferation in the olfactory bulb and hippocampus [22;23]. Consequently, as a regulator of neurogenesis, NPY may have a significant role in the development and/or treatment of mood disorders. Due to the lack of clinical efficacy in currently approved drugs for some patients, NPY represents a novel approach in the search and development for more effective antidepressants.

1.2 Objective

The aim of this study was to elucidate the role of NPY receptor subtypes in emotional behavior using NPY transgenic and knockout animals and to assess NPY as a modulator of neurogenesis, in animal models. This was accomplished through four main objectives. The first objective involved measuring emotional behavior (both anxiety and depression) in Y_1 and Y_2 receptor knockout mice. The second objective was to examine neurogenesis (specifically proliferation) in Y_2 receptor knockout mice. The third and forth objectives were to determine the effect that chronic treatment with NPY has on emotional behavior and neurogenesis (proliferation, survival, and differentiation) in the rat. In addition, the anxiety profile of the NPY transgenic rat was also examined to determine the effect that overexpression of hippocampal NPY has on this behavior.

1.3 Hypothesis

There is preclinical and clinical evidence that NPY is involved in the regulation of emotional behaviors. Using NPY transgenic and knockout animal models, the role of the Y_1 and Y_2 receptor subtypes in anxiety and depression-like behaviors will be further classified. There is also evidence that chronic depression is associated with neuronal loss and abnormalities of neurogenesis in the hippocampus that can be reversed with chronic antidepressant treatment. It has been shown that NPY promotes neurogenesis and neuroproliferation in the CNS including in the olfactory bulb and dentate gyrus of the hippocampus. Thus, assessment of NPY induced neurogenesis in the subgranular zone (SGZ) of the dentate gyrus of the hippocampus in relation to emotional behavior in animal models should determine the possible efficacy of NPY and its receptor subtypes as potential antidepressant therapies.

1.4 Rationale: definition, prevalence, medical and economic impact of depression

Major depressive disorder is characterized by symptomatic criteria such as depressed mood, low self esteem, feelings of hopelessness, worthlessness and guilt, as well as recurrent thoughts of death and suicide as described in the Diagnostic and Statistical Manual [24]. The diagnosis for major depression is given when symptoms persist for longer than a two week period of time and normal social and occupational function is disrupted. Worldwide, an estimated 121 million people suffer from depression [25]. It was the leading cause of disability (years living with the disease) and the 4th leading contributor to the global burden of disease (disability adjusted life years; DALYs) in 2000 [26]. Studies have consistently documented higher rates of depression among women (9.5%) than among men (5.8%) in any given year [26]. By the year 2020, depression is projected to reach 2nd place of the ranking of DALYs calculated for all ages and both sexes [26]. In Canada, approximately 7.9% to 8.6% of adults will experience major depression at some time in their lives [27]. Approximately 1%-2% of the population is afflicted with bipolar disorder (previously described as manic-depressive disorder) in which men and women are affected equally [28]. Suicide is estimated to be the cause of death in up to approximately 15% of individuals with major depressive and bipolar disorder [29]. In addition to the emotional consequences associated with depression, there are financial considerations as well. The costs of disability and premature death associated with depressive illness results in an economic burden of billions of dollars worldwide [26]. In Canada alone, it is estimated that 14.4 billion per year is the cost of mental health disorders, including depression, to society [30]. Hospitalization represents between 43% and 75% of the average per patient cost

[30]. In relation to major chronic diseases such as Alzheimer's disease, cancer, and schizophrenia; depression is ranked third by prevalence and sixth in terms of economic burden [31]. Thus, the emotional and financial impact of depression on society is a major motivation for the research and treatment of this disorder.

1.4.1 Current antidepressant treatment strategies

The development of current antidepressant treatment strategies resulted from the accidental discovery in 1957 that tuberculosis patients treated with iproniazid often felt "too well" which led to behavior in which patients "...failed to observe ordinary precautions, overexerted themselves, or discontinued treatment prematurely" [32-34]. Despite the perceived detrimental side effect of the drug, iproniazid was found to somewhat inhibit the effects of the enzyme that blocked the inactivation of noradrenaline, monoamine oxidase [32]. By the end of the 1950s, the monoamine oxidase inhibitors (MAOI) were on the market for the treatment of mood disorders [35]. Non-selective inhibitors of monoamine uptake, the tricyclic/tetracyclic antidepressants (TCA) were also discovered by chance from antihistamine research [36;37]. The efficacy of MAOI and TCA drugs led to the theory that depression is caused by a deficit and mania is caused by an excess of monoamines such as noradrenaline and serotonin in the synaptic cleft [38].

Current antidepressant medications that increase serotonergic and noradrenergic neurotransmission are the most common and the most effective treatments [39]. Standard pharmacotherapy relies on a broad class of antidepressants known as the selective serotonin (5-hydroxytryptamine or 5-HT) reuptake inhibitors (SSRI), noradrenaline (NA) reuptake inhibitors (NARI) and combined noradrenergic and specific serotonergic antidepressants (NASSA). The acute mechanism of action of traditional antidepressants involves the inhibition of monoamine reuptake from the synaptic cleft [39]. However, all

available antidepressants exert their mood elevating effects after chronic (several weeks or months) administration, not acute treatment [40]. The other major drawback to the monoamine hypothesis includes the success of drugs like tianeptine; a selective serotonin reuptake enhancer (SSRE) whose acute biochemical action is to enhance; rather than inhibit, serotonin reuptake [41;42].

Although approximately 30-40% of patients demonstrate complete remission and many patients show partial responses under current antidepressant strategies [43]. Approximately 30-40% of patients fail to achieve complete remission [43], up to 20% have not recovered 2 years later [44;45], and 10% remain depressed despite multiple interventions [46;47]. Clinical trials demonstrate that 30-40% of depressed patients fail to respond to first-line antidepressant treatment despite adequate compliance, dose, and duration [46;48;49]. Additional strategies such as electroconvulsive therapy (ECT) are very effective in patients who are resistant to antidepressant medication [50]. However, there is a very low patient acceptability due to the perceived aversive nature of the procedure and the significant effects on cognitive and memory function following treatment [51]. Consequently, the significant percentage of treatment-resistant patients necessitates the requirement for research directed towards novel mechanisms of pharmacological intervention and the elucidation of the pathophysiology of mood disorders. The most recent evidence demonstrates that impairment of neuroplasticity (specifically hippocampal neurogenesis) may at least partly underlie the pathophysiology of mood disorders and that antidepressants and mood stabilizers exert major effects on signaling pathways that regulate hippocampal neurogenesis and cell survival [52-56].

1.4.2 Neurogenesis and depression

Clinical evidence has revealed that chronic depression is associated with neuronal loss and gray matter alterations [57]. In human magnetic resonance imaging [MRI] studies, a correlation between reduced hippocampal volume and recurrent major depression in women that is unrelated to age was discovered [58]. This discovery was confirmed by several studies that found an overall decrease in the hippocampal volume of depressed patients compared to healthy controls [59;60]. Interestingly, the smaller hippocampal volumes observed in chronically depressed patients were not found in recovered patients [61]. It is unclear if a lower hippocampal volume leads to the development of depression, results from, or is simply an epiphenomenon of the disease. However, the fact that successful antidepressant treatments including pharmacotherapy (MAOI, [40] TCA, [62] SSRI and SNRI, [40;62-64] the SSRE tianeptine, [41;42] the mood stabilizer lithium, [65]) as well as ECT [66] reverse this abnormality strongly supports the possibility that a reversal of deficits in the regulation of neuroplasticity are involved in the successful treatment of depression.

1.4.3 The regulation of neurogenesis and antidepressant treatment

The discovery of neural stem cells in adult rodents, primates and humans revolutionized attitudes towards the function of neurons within the CNS [67-70]. It was discovered that new neurons could be made from existing progenitor cells [67-70]. The primary progenitor cells are considered to be neural stem cells with astroglial characteristics [71]. These primary progenitor cells have been shown to display undifferentiated, self-renewable, and multipotent features and are the precursors to multipotent secondary progenitors that give rise to a precursor committed to a specific lineage in the CNS [71]. It is generally accepted that new neurons are born in two distinct

regions of the CNS; the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus. In the SVZ, most new neurons migrate anteriorly through the rostral migratory stream into the olfactory bulb where they mature into interneurons [71]. In the SGZ, progeny migrate outward to the granular cell layer and differentiate into neurons [72]. The neuron matures in the granule cell layer as extends its axons outward to the CA3 region of the hippocampus [72]. The process of neurogenesis is defined in terms of proliferation (one round of cell division), migration, differentiation into neuronal or glial phenotypes and survival [73]. There are approximately 1,000 to 3,000 new neurons made each day in mice and rats in the dentate gyrus and SVZ [70]. Recent studies reveal that new neurons born during adulthood become integrated into circuits, survive to maturity, and may permanently replace neurons born during development [74].

There is considerable evidence that a wide range of antidepressant treatments increase the proliferation (but not survival or differentiation) of newly born neurons in the SGZ of the dentate gyrus (but not in the SVZ). For example, rats given the SSRI fluoxetine systemically for 3 weeks experienced a 70% increase in the number of cells produced in the dentate gyrus [75]. Various antidepressant treatment strategies including pharmacotherapy (MAOI, [40] TCA, [62] SNRI, [40;62-64] atypical antidepressants such as the SSRE tianeptine, [41] the mood stabilizer lithium, [65]) as well as ECT [66] and physical activity [76] have all been shown to stimulate progenitor cell proliferation in the dentate gyrus of mice, rats, or primates. Most interestingly, blocking neurogenesis with x-rays aimed at the SGZ in the hippocampus to kill stem cells that give rise to new neurons

also block the positive behavioral effect of chronic antidepressant treatment in the novelty-suppressed feeding test [62].

The theory that deficits in hippocampal neurogenesis are causally related to the development of depression is supported by evidence that suggests prolonged stress and anxiety (two well known contributors to the development of depression) can lead to decreased hippocampal proliferation [77]. For example, chronic restraint stress [78]; exposure to predator odor [79;80]; and prenatal stress [81] in rodents have all been shown to decrease hippocampal proliferation. Psychosocial stress and social defeat in marmosets and tree shrews [41;82;83] have also been shown to decrease hippocampal proliferation. Interestingly, the atypical antidepressant tianeptine can reverse this stress-induced reduction [41]. The learned helplessness model of depression (in which animals are exposed to an inescapable shock) can also produce significant reductions in hippocampal proliferation with concomitant behavioral deficits [63]. It was also demonstrated that the behavioral deficits and reduction in dentate gyrus proliferation could be reversed with antidepressant treatment [63].

Based on these studies, there seems to be a strong interaction between stress, depression, antidepressant treatment and hippocampal proliferation. Interestingly, there is also support for NPY as a neuromodulator of emotional behavior and neurogenesis in the hippocampus. The evidence for NPY and its receptor subtypes in emotional behavior including both anxiety and depression will be discussed in chapters three and four. Chapter two will first introduce NPY and review the distribution of NPY and NPY receptor subtypes in the CNS as well as the pharmacological tools used to characterize this peptide.

Chapter 2: Distribution and pharmacological characteristics of NPY receptors

NPY was isolated from porcine brain more than two decades ago [1]. NPY shares high sequence homology and structural identity with peptide YY (PYY) and the pancreatic polypeptides (PPs) [1]. In the rat brain, NPY- like immunoreactivity (NPY-IR) is concentrated in the neocortex, basal forebrain, striatum, hippocampus, amygdala, hypothalamus, brain stem [2]. At present, five NPY receptor subtypes have been cloned and designated Y_1, Y_2, Y_4, Y_5 and y_6 [8-10]; all of which couple to G_i proteins and inhibit the production of cyclic AMP [11]. In the rat brain, localization of Y_1 receptor mRNA [84] closely matches that of the Y_1 receptor protein [9;85] with predominant expression in the cerebral cortex, thalamus and brainstem nuclei [9]. NPY Y_2 receptor mRNA and protein are abundantly expressed in the hippocampus and brainstem, while moderate levels of Y_2 receptors are detected in the hypothalamus [9;84;85]. Only low levels of Y_4 receptor mRNA expression have been detected thus far in the rat CNS [84]. NPY Y_5 receptor mRNA is expressed in the external plexiform layer of the olfactory bulb, anterior olfactory nuclei, hippocampus, suprachiasmatic and arcuate nuclei [84]. The y_6 receptor is not expressed in the rat [86], while in human and primates, the cDNA contains a single base deletion resulting in the expression of a non-functional NPY receptor [87]. (Please see *Table 2.1* for NPY receptor subtype expression in the CNS)

NPY and PYY have high affinity for the Y_1, Y_2, and Y_5 receptor subtypes [88]. Truncated carboxy-terminal fragments of the NPY and PYY peptides bind with high affinity to the Y_2 and Y_5 receptor subtypes [88;89]. The Y_4 receptor has high affinity for PPs but lower affinity for NPY and PYY [90]. The Y_1 receptor agonists include [Leu^{31},Pro^{34}]NPY, [Leu^{31},Pro^{34}]PYY, [Pro^{34}]NPY, and [Pro^{34}]PYY [91;92].

[Leu^{31},Pro^{34}]NPY and [Leu^{31},Pro^{34}]PYY also have affinity for the Y_4 and Y_5 subtypes [88]. The Y_2 agonists include [Ahx$^{5-24}$, γ-Glu^{2}-ε-Lys^{20}]NPY [93] and C2-NPY [94;95]. GR231118 is an agonist for Y_4 receptors [96;97]. Additional agonists for the Y_5 receptors include [cPP(1–7),NPY(19–23),Ala^{31},Aib^{32},Gln^{34}]hPP [98], [Ala^{31},Aib^{32}]NPY [99], and [hPP(1-17), Ala^{31},Aib^{32}]NPY [98]. The Y_1 receptor antagonists include BIBP3226 [100], BIBO3304 [101], GR231118 [102], J104870 [103], J115814 [104], and GI264879A [105]. The Y_2 receptor antagonists include BIIE0246 [106] and the novel compound JNJ-5207787 [107]. CGP71683A [108] and L152804 [109] are Y_5 antagonists.

Additional tools to characterize NPY receptor subtypes include the radioligands. For example the [^{125}I]-PYY targets all NPY receptor subtypes while [^{125}I]-[Leu^{31},Pro^{34}]PYY labels the Y_1, Y_4, and Y_5 receptors [110]. Additional radioligands exist for the Y_1 ([^{125}I]-GR231118 [111;112]), Y_2 ([^{125}I]-PPY(3-36) [110]), Y_4 ([^{125}I]-PPs [113], [^{125}I]-GR231118 [111;112]), and Y_5 [^{125}I]hPP [9], [^{125}I]-[hPP$_{1-17}$, Ala^{31},Aib^{32}]NPY [114], [^{125}I]-[cPP(1–7), NPY(19–23),Ala^{31},Aib^{32},Gln^{34}]hPP [115]) receptor subtypes. (Please see *Table 2.2* for pharmacological characteristics of NPY receptor subtypes)

NPY receptor subtypes have several physiological functions. For example, NPY is one of the most potent peptides to induce feeding by activating Y_1 and/or Y_5 subtypes [116;117]. Studies in knockout and transgenic animals have revealed that NPY is implicated in alcohol intake (mediated by the Y_1 subtype) [118] and seizure activity (mediated by Y_1, Y_2, and Y_5 subtypes) [117;119]. There is also evidence that Y_2 receptor subtypes have a role in modulation of learning and memory processing [120]. Furthermore, NPY Y_1 receptors have been associated with depression [18] and anxiety related behaviors [121].

Table 2.1: NPY peptide and NPY receptor subtype expression in the CNS

NPY	Y_1	Y_2	Y_4	Y_5
Neocortex, hippocampus, basal forebrain, striatum, amygdala, hypothalamus, and brain stem	Cerebral cortex, thalamus, and brain stem	Hippocampus, brain stem nuclei, and hypothalamus	Very low in brain paraventricular hypothalamus, and interpeduncular nucleus	Hippocampus, plexiform cortex of the olfactory bulb, suprachiasmatic and arcuate nuclei

Table 2.2: Pharmacological characteristics of NPY receptor subtypes

	Y_1	Y_2	Y_4	Y_5
Preferred Endogenous Ligand	Neuropeptide Y (NPY), Peptide YY (PYY)	NPY, PYY, NPY(3-36), PPY(3-36)	Pancreatic Polypeptides (PPs)	NPY, PYY, NPY(3-36), PPY(3-36)
Agonists	[Pro^{34}]NPY, [Pro^{34}]PYY, [Leu^{31},Pro^{34}]NPY, [Leu^{31},Pro^{34}]PYY,	NPY(13-36), PPY(13-36), [Ahx$^{5-24}$, γ-Glu^{2}-ε-Lys^{20}]NPY, C2-NPY	[Leu^{31},Pro^{34}]NPY, [Leu^{31},Pro^{34}]PYY, GR231118 1229U91	[Leu^{31},Pro^{34}]NPY, [Leu^{31},Pro^{34}]PYY, [Ala^{31},Aib^{32}]NPY, [hPP(1-17), Ala^{31},Aib^{32}]NPY d-Trp^{32}-NPY, [cPP(1–7),NPY(19–23),Ala^{31},Aib^{32},Gln^{34}]hPP
Antagonists	BIBP3226, BIBO3304, GR231118, J104870, J115814, GI264879A,	BIIE0246 JNJ-5207787	-	L152804, CGP71683A
Radioligands	[^{125}I]-PYY, [^{125}I]-[Leu^{31}, Pro^{34}]PYY, [^{125}I]-GR231118	[^{125}I]-PYY, [^{125}I]-PPY(3-36)	[^{125}I]-PPs, [^{125}I]-PYY, [^{125}I]-[Leu^{31},Pro^{34}]PYY, [^{125}I]-GR231118	[^{125}I]-PYY, [^{125}I]-[Leu^{31},Pro^{34}]PYY, [^{125}I]hPP [^{125}I]-hPP(1-17), Ala^{31},Aib^{32}]NPY, [^{125}I]-[cPP(1–7),NPY(19-23),Ala^{31},Aib^{32},Gln^{34}]hPP

Chapter 3: Role for NPY and its receptor subtypes in anxiety

3.1 The effect of stress on central NPY signaling

NPY is considered an important neuromodulator in the regulation of anxiety-related behaviors. Several studies have shown that exposure to acute and chronic stress paradigms such as physical immobilization produces widespread changes in NPY expression throughout the CNS. For example, 1 hour of restraint stress in Sprague-Dawley rats decreased NPY mRNA levels and NPY-IR by 30% in the amygdala [122]. Two hours following restraint stress, NPY mRNA decreased by 35% in the neocortex and amygdala. However, 10 hours after restraint stress, NPY-IR increased 23% in the hypothalamus [122]. Another study looked at repeated stress in Wistar-Kyoto rats [123]. After 1 hour of restraint stress, NPY mRNA increased in the arcuate nucleus of the hypothalamus by 81%, but after 3 days (1 hour each day), NPY mRNA increased by 40% in the arcuate nucleus and 50% in the medial amygdala (MeA) [123]. After chronic restraint (1 hour/day for 9-10 days), there was an up-regulation of prepro-NPY mRNA and NPY itself in the amygdala but not in hypothalamic or cortical regions [124]. Consequently, acute physical restraint, which promotes experimental anxiety, primarily suppressed NPY expression but chronic physical restraint generally enhanced NPY signaling, especially in the amygdala. These results are believed to support an early hypothesis that suggests NPY may act to "buffer" the behavioral effects of stress-promoting signals [125].

3.2 The effect of exogenous NPY on anxiety-related behaviors

NPY is consistently implicated in the pathogenesis of anxiety disorders, based on a significant number of findings that show NPY-induced anxiolytic activity in animal models widely used for the screening of anxiolytic compounds. NPY is reported to elicit

anxiolytic-like effects in models of anxiety including exploratory behavior-based tests such as the open field, elevated plus-maze, and light/dark compartment test [126;127], social interaction [128], punished responding tests, [129] and fear-potentiated startle [130].

The open field test is often used to measure behavioral changes induced by anxiolytic or anxiogenic-like compounds in animal models [131]. Anxiolytic drugs such as benzodiazepines and barbiturates increase the number of entries and time spent in the central area of the arena [131]. Total crossings are presented as a measure of general locomotor activity in the arena. At higher doses, traditional anxiolytic drugs have significant sedative effects and suppress locomotor activity [132]. Similarly, NPY causes dose-dependent suppression in open field activity when the intracerebroventricular (icv) dose of NPY exceeds 5 μg [133-135]. The significant effect of NPY on sedation in the open field led investigators to examine the potential anxiolytic properties of this peptide in the elevated plus-maze.

The elevated plus-maze is one of the most widely used tests for anxiety in animal models [136]. The test is based on the conflict between the natural aversion of rodents for open spaces and the drive to explore a novel environment [136]. Typically, the time spent on the open arms and the numbers of entries onto the open and closed arms of the maze are recorded. The percentage of open arm entries relative to the number of total arm entries is considered to be the superlative measure reflecting innate fearfulness [136]. NPY, administered icv, decreased the preference for closed arm entries and increased the time spent on open arms [121]. Higher doses of NPY (exceeding 2 nmol) suppressed

entries into both closed and open arms, consistent with the sedative action of NPY observed at high doses in the open field [133].

A central mechanism involving the NPY Y_1 receptor subtype in the anxiolytic effect of NPY is supported by several pharmacological studies. For example, icv administration of an antisense oligonucleotide targeted at Y_1 receptor mRNA, attenuated NPY-induced anxiolytic-like effects in the elevated plus-maze [137] and blocked the anxiolytic-like effect of NPY in the central nucleus of the amygdala (CeA) in the same test [138]. This was confirmed in a study that demonstrated anxiolytic-like activity of the NPY, PYY, and the $Y_1/Y_4/Y_5$ agonist [Leu^{31}, Pro^{34}]NPY but not the Y_2 agonist NPY(13-36) [130]. Further support for the Y_1 receptor was revealed with the anxiogenic-like effect of the Y_1 receptor antagonist BIBP3226 [139]. The Y_2-type receptor agonist, NPY(13-36), was later shown to induce anxiogenic-like effects in this model, using mice, when administered icv [140].

More recently, there was an attempt to confirm the receptor subtype(s) involved in the anxiolytic/anxiogenic action of NPY as well as determine which receptor(s) are involved in the sedative action of NPY. This study examined icv injection of NPY as well as specific receptor agonists for the Y_1 receptor ([D-His^{26}]NPY), Y_2 receptor (C2-NPY), and Y_5 receptor ([cPP(1–7),NPY(19–23),Ala^{31},Aib^{32},Gln^{34}]hPP) in the elevated plus-maze and open field tests [141]. The results revealed that NPY and the Y_1 agonists had a dose-dependent anxiolytic-like effect in both behavioral tests. However, in contrast to NPY, which caused significant sedation in the open field test, the Y_1 agonist was without sedative effect [141]. The Y_2 agonist showed neither anxiolytic-like nor sedative effects and the Y_5 agonist showed anxiolytic-like activity in both behavioral tests and caused

sedation in the same dose range as NPY in the open field test [141]. The authors conclude that the anxiolytic-like effects of icv-administered NPY in rats are mediated via both Y_1 and Y_5 receptors, whereas sedation is mediated via Y_5 receptors [141].

Additional support for the anxiolytic-like effect of NPY has been confirmed in the light/dark compartment test where icv NPY increased the number of transitions between the two compartments [127], a validated measure of anxiolytic activity in this test. Social interaction has also been pharmacologically validated as an experimental model of anxiety [142;143]. The time spent in active social behavior, as well as locomotor activity, is recorded [142]. NPY, when microinjected into the basolateral nucleus of the amygdala (BLA) [144] and into the caudal dorsolateral septum [145] increases social interaction. Thus, NPY-induced anxiolysis in this paradigm of anxiety appears to be mediated by several brain regions. As in the elevated plus-maze, involvement of the Y_1 receptor in social interaction is supported by blocking the anxiolytic-like effect of NPY with intra-amygdalar injection of the selective Y_1 receptor antagonist, BIBO3304 [144]. The role of the Y_5 receptor was also shown using [cPP(1–7),NPY(19-23),Ala^{31},Aib^{32},Gln^{34}]hPP, a Y_5 receptor agonist. This agonist and the mixed Y_5/Y_2 agonist NPY(3–36) caused anxiolytic-like effects when injected into the BLA. The effect of NPY(3–36) was blocked by pretreatment with a novel Y_5 antagonist, Novartis 1, synthesized by Eli Lilly [146]. However, the Y_5 antagonist CGP71683A did not influence anxiety in any exploratory model of anxiety [147].

It has also been suggested that the Y_2 NPY receptor may mediate anxiogenic-like behaviors in the amygdala in this task [146]. One study found a dose-dependent decrease in the time spent in social interaction when the Y_2 receptor agonist, C2-NPY, was directly

injected into the BLA [146]. However, the authors did not attempt to reverse the anxiogenic-like effects of this molecule with a selective Y_2 receptor antagonist, such as BIIE0246 [106]. The Y_2 receptor agonists may have an anxiogenic effect because activation of the presynaptic Y_2 receptor may lead to decreased release of NPY [148] and a subsequent decrease in Y_1 receptor activation. Overall, in the exploratory tests of anxiety including the open field, elevated plus-maze, and social interaction, NPY produces robust anxiolytic-like activity that is mediated through the Y_1 (and possibly Y_5) receptor subtypes while the anxiogenic effects of NPY are mediated through Y_2 receptors.

Several versions of operant behavioral analysis for anxiety have been developed such as the Vogel conflict test and Geller-Seifter test that are based on conflict of motivations rather than unconditioned exploratory models. In these tests, the subject experiences opposing and concomitant tendencies of desire (for example, to obtain a reward) and of fear (avoidance of a potentially aversive stimulus) [149]. In the Vogel conflict test, water-deprived rodents are exposed to mild and intermittent electric shock via the spout of a water bottle when attempting to drink [150]. In this test, treatment with anxiolytic drugs increases the number of accepted shocks during the punished phase. It has been shown that icv NPY markedly increased the number of electric shocks accepted in this test [121]. At the doses employed, NPY was reported not to affect pain sensitivity in a shock threshold test, or thirst. Thus, the anti-conflict effects are considered to be related to a reduction of anxiety [121]. In the Geller-Seifter conflict test, rats are trained to respond for a food reward and are exposed to a modest electric shock during the "conflict" component of the procedure [129]. ICV injection of NPY consistently produces dose-

dependent anti-conflict/anxiolytic-like effects in the Geller-Seifter test [129;151;152], an established animal model of anxiety especially suitable for detecting the effects of benzodiazepine-like anxiolytics. The Y_1 receptor subtype is implicated in this test as well. Intra-amygdalar injection of [Leu^{31}, Pro^{34}]NPY was shown to have anxiolytic-like effects [153]. As in the exploratory models of anxiety, NPY can also induce anxiolysis in the conflict-based tests.

Startle is an adaptive response to acoustic stimuli that enables the individual to avoid, or reduce, the risk of an injury by a predator [154]. In fear-potentiated startle, an acoustic stimulus is paired with an aversive intervention such as foot-shock or air-puff, and after training, the stimulus alone is capable of elevating startle amplitude [154]. As in the elevated plus-maze, icv injections of NPY, PYY, and the $Y_1/Y_4/Y_5$ agonist [Leu^{31}Pro^{34}]NPY inhibited fear-potentiated startle, whereas the Y_2 agonist NPY(13-36) had no effect [130]. The Y_1 receptor is consistently indicated across diverse animal models of anxiety to mediate the anxiolytic-like activity of NPY. However, the Y_5 receptor cannot be ignored because [Leu^{31}Pro^{34}]NPY does have some efficacy for the Y_5 receptor subtype [116]. In addition, the Y_5 –Y_2 differentiating receptor agonist NPY(2-36) has shown anxiolytic-like activity in the elevated plus maze and fear-potentiated startle [130].

3.3 Anxiety-like behavior in NPY receptor knockout and transgenic animals

The development of the NPY transgenic rat [155] has provided a unique opportunity to study the effects of this peptide on anxiety-related behaviors. In this transgenic rat model, there is central overexpression of prepro-NPY mRNA and NPY peptide in the CA1 region of the hippocampus and decreased Y_1 binding sites within the hippocampus (CA1, CA2, and dentate gyrus) [156]. Recently, NPY protein levels were also shown to

be significantly higher in the paraventricular, suprachiasmatic and supraoptic nuclei of the hypothalamus and tended to be increased in the arcuate nucleus in these rats [13;157]. The transgenic animals were generated using a 14.5-kb fragment of the rat NPY genomic sequence that includes the normal intronic sequence elements and is flanked by a 5-kb 5' sequence thought to contain the major regulatory elements that normally control NPY expression [155]. Consequently, the regulation of the NPY transgene is predicted to be similar to the regulation of endogenous NPY. These molecular and neurochemical events led to an altered anxiety profile in NPY transgenic rats that included an insensitivity to restraint stress in the elevated plus-maze and an increase in the number of punished drinking events in the Vogel conflict test [156]. The anxiolytic-like profile of the NPY transgenic rat was replicated in a study that showed NPY transgenic animals were resistant to acute physical restraint stress measured by the elevated-plus maze and displayed anxiolytic-like activity in the open field (Please see *Appendix A)* [158]. The behavioral profile observed in the NPY transgenic rats was not associated with any significant changes in corticosterone levels before or following a stress challenge [156]. This suggests a mechanism other than hypothalamic-pituitary-adrenal axis modulation and supports the role of the limbic system in the modulation of anxiety-related behaviors by NPY.

The central role of the limbic system is also supported by recent studies involving Y_2 receptor subtype knockout ($Y_2^{-/-}$) mice. Mice deficient in the Y_2 receptor subtype displayed an anxiolytic-like phenotype in the elevated plus-maze and open field test [159]. More recently, NPY $Y_2^{-/-}$ mice displayed increase preference for the central area of the open field when compared to wildtype control ($Y_2^{+/+}$) animals without changes in

locomotor activity [159]. These findings have been confirmed by an independent laboratory using the open field, elevated plus-maze, and light-dark compartment test [160]. These receptors are considered to be autoreceptors that provide negative feedback to NPY-ergic nerve terminals to modulate NPY release [89]. Consequently, $Y_2^{-/-}$ mice are predicted to have increased endogenous peptide expression, analogous to the phenotype of NPY transgenic rats, that may contribute to the underlying mechanism responsible for anxiolytic-like behaviors regulated by NPY [159].

Interestingly, NPY knockout ($NPY^{-/-}$) mice did not show a dramatic change in anxiety-like behavior in the elevated plus-maze [161]. $NPY^{-/-}$ mice were found to have a significant increase in the amplitude response in the acoustic startle test and they were less active in the central part of the open field, suggesting that these animals were more anxious [161]. Overall, the results from NPY transgenic and knockout animals provide compelling evidence for the role of NPY in the modulation of anxiety.

3.4 Clinical evidence for NPY in anxiety disorders

In addition to the extensive preclinical data supporting NPY in the regulation of anxiety-like behaviors, a number of clinical studies have been published on the subject. In human subjects, there is a positive association between acute, uncontrollable psychological stress and robust increases in plasma levels of NPY [162;163]. The increase in plasma NPY was positively correlated with increased cortisol and norepinephrine concentrations [162;163]. A correlation between higher levels of stress-related NPY release and lower levels of subjective psychological distress was also found [162], supporting the possibility that NPY exhibits anxiolytic activity during stress in human subjects [162].

3.5 How does NPY regulate emotionality?

The mechanistic action of NPY in anxiety-related behaviors is strongly associated with the amygdala. The amygdala is a key structure to the regulation of anxiety and expression of emotional responses to stress [164;165]. Attention has been focused particularly on the basolateral complex (lateral, basolateral, and basomedial nuclei) and CeA [164;165]. The amygdala contains NPY [2] and significant levels of Y_1 and Y_2 receptor subtypes [9;85]. These areas may be potential neural substrates to the behavioral effects of NPY on emotional regulation. NPY is also produced by neurons in the hippocampus with considerable expression of the Y_1 and Y_2 receptor subtype [119;166;167]. In addition to the substantial evidence for the involvement of the amygdala and hippocampus, there is also evidence for the periaqueductal gray [128;168], septum [145], and locus coeruleus [169]. However, the specific contribution of these anatomical regions requires further elucidation.

The interaction between corticotrophin-releasing factor (CRF) and NPY has been proposed as a means in which emotional behavior is regulated (For review see [170]). CRF pathways are known to strongly influence anxiety and stress-related behaviors [171]. For example, icv administration of CRF increases anxiety-like behavior and CRF antagonists block the effects of stressful events [172]. This has been confirmed in studies using CRF-overexpressing and CRF knockout mice [173;174]. It has been shown that NPY can counteract the anxiogenic effect of CRF in the hippocampus [175], hypothalamus [176], the locus coeruleus [177], the periaqueductal gray [128;177], and the septal nucleus [145]. It is interesting to note that deletion of Y_2 receptors causes a 60% reduction in CRF mRNA expression [178] that might contribute to the anxiolytic-like behavior of NPY Y_2 receptor knockout mice [160]. Support for this hypothesis was

demonstrated in a study that utilized neonatal thyroxine treatment (a model of hyperthyroidism) [179]. Thyroxine treated adult animals displayed reduced anxiety in the motility box and elevated plus-maze, a reduction in the number of CRF-IR neurons in the CeA, as well as an increase in the number of NPY-IR neurons in nuclei of the basolateral complex of the amygdala [179].

It has also been proposed that NPY increases GABA signaling to produce anxiolysis; analogous to many classes of anxiolytic drugs like benzodiazepines [180]. NPY is localized in GABAergic neurons in the amygdala [181] and there is evidence that NPY may directly modulate the activity of GABAergic neurons by stimulating Y_1 receptors [139]. It has been shown that diazepam counteracts the anxiogenic effect of the Y_1 receptor antagonist BIBP3226 [139], suggesting that the equilibrium between GABA-ergic and NPY-ergic neurotransmission may be important for the regulation of the emotional state in animal models [182]. This conclusion is supported by a recent study that found chronic treatment with positive (anxiolytic) or negative (anxiogenic) regulators of $GABA_A$ receptors modulate Y_1 receptor-mediated transmission in the amygdala [183]. The authors suggest that the NPY-Y_1-mediated transmission and the GABAergic system may act together on the same postsynaptic target sites to decrease anxiety and that the Y_1 receptors might also play a role in controlling both the NPY and GABA presynaptic release [184].

However, it was recently shown that the anxiolytic-like effect produced by inhibiting the metabotropic glutamate receptor 5 [mGlu5] with the mGlu5 receptor antagonist MPEP is mediated by the NPY receptor, and not GABA [185]. In order to determine the mechanism that contributes to the anxiolytic action of MPEP, the authors injected either

the benzodiazepine antagonist flumazenil or the Y_1 receptor antagonist BIBO3304 into the BLA. Flumazenil antagonizes the effect of the several different classes of anxiolytic agents including the benzodiazepine diazepam, serotonergic agents [186] and noradrenergic ligands [187] in animals models of anxiety through a GABA mediated mechanism [188]. However, the anxiolytic effects of MPEP were not changed by flumazenil, but were abolished by BIBO3304 administration [185]. The authors also found a decrease in NPY-IR neurons after three doses of MPEP administration, which suggests a decrease in NPY levels in the amygdala. The authors propose that the decrease in NPY levels may result from enhanced release of peptide and that this release is responsible for the anxiolytic action of MPEP [185]. Interestingly, administration of metabotropic glutamate receptor antagonists into the hippocampus also produces anxiolytic-like effects [189]. NPY has been shown to inhibit glutamate release in the hippocampus [190;191] leading to the speculation that the anxiolytic-like effect of hippocampal NPY overexpression in NPY transgenic rats resembles the anxiolytic-like action of the metabotropic glutamate receptor antagonists. The interaction between glutamate acting via mGlu5 receptors and NPY may represent a novel mechanism of anxiolytic action in the brain, independent of the traditional GABA mediated benzodiazepine signaling [185].

Overall, these studies demonstrate that NPY and its receptor subtypes are significantly involved in the regulation of anxiety-like behaviors in both animal models and human subjects.

Chapter 4: Role for NPY and its receptor subtypes in depression

4.1 The effect of animal models of depression on central NPY signaling

Preclinical data has consistently indicated a role for NPY in depression. Such studies have employed animal models widely considered to mimic, at least in some respects, the behavioral, biochemical and neurochemical aspects of the clinical condition. The animal models are diverse and include the Flinders Sensitive Line (FSL), a unique group of selectively inbred rats that display features similar to those observed in depressed patients including reduced basal motor activity [192], elevated REM sleep [193] and increased immobility and anhedonia responses after stress exposure [194]. NPY-IR and NPY Y_1 receptor binding sites were shown to be differentially altered, depending on the brain region studied, in the FSL rats [195;196] but not in the control Flinders Resistant Line (FRL). NPY Y_2 mRNA expression was unchanged, suggesting that this subtype may not play such an important role as Y_1 receptors in this model. Moreover, treatment with the selective serotonin reuptake inhibitor (SSRI) fluoxetine, attenuated changes in NPY receptor mRNA observed in the FSL animals [195].

Chronic mild stress (CMS) is an animal model of depression that exposes animals to a variety of mild stressors including food and/or water deprivation, overnight illumination, cold immersion, soiled cage, cage tilt, and noise, over a period of weeks or months [197] [198]. The CMS model was created as a method for mimicking anhedonia [198;199], defined as a diminished responsiveness to rewards and measured as decreased intake of a sucrose solution [199]. A recent study demonstrated that CMS produced significant decreases in NPY expression in several hypothalamic and thalamic areas [200]. Another study reported decreases in NPY mRNA in the hippocampal dentate gyrus and increases in the arcuate nucleus [201].

The olfactory bulbectomy (OBX) in the rat is an animal model of depression that was originally created from the theory that depression is a biochemical disorder and develops in individuals who are predisposed due to neuronal regulatory deficits [202]. The olfactory bulb has been implicated as a major contributor to the etiology of depressive states in rodents. Olfactory bulb neurons interconnect extensively with limbic structures including the piriform and dentate gyrus and receive direct projections from the serotonergic raphe nuclei and the noradrenergic locus coeruleus [203]. It is predicted that bilateral aspiration of the olfactory bulbs deprives rodents of their primary sensory modality and strongly effects their interaction with the external environment [204]. The behavioral effects of OBX include hyper-locomotion, deficits in defensive freezing, deficits in learning and memory, hyper-reactivity to noxious stimuli and stress, loss of circadian activity/rhythms, anhedonia, alterations in feeding patterns, as well as decreased weight gain and abnormal sexual behavior [205]. This animal model is valuable given that it mimics some characteristics of the human disease. For example, chronic, rather than acute treatment with antidepressants is required to reverse the behavioral effects of OBX [206]. This model provides strong theoretical rationale and similar phenomenology between animal models and humans suffering from depressive symptoms. A role for NPY in depressive disorders is found in studies using the OBX model. Sub-chronic icv administration of NPY attenuated increases in ambulation, rearing, grooming and defecation scores consistently found when OBX animals are tested in the open field [12]. Treatment with NPY also increased noradrenaline and serotonin levels in the amygdala and hypothalamus [12]. NPY also reversed the suppression of lymphocyte proliferation seen following OBX [12] as well as in depressed patients [207].

Another study demonstrated that OBX caused long term increases in NPY gene expression in the olfactory/limbic system. Prepro-NPY mRNA levels in the piriform cortex and dentate gyrus were significantly elevated in bulbectomized rats 14 and 28 days (but not 3 or 7 days) after surgery compared to sham-operated and surgically naïve rats; suggesting that NPY plasticity may play some role in this model [205]. The increase in prepro-NPY in the piriform cortex after OBX was confirmed in a later study that also revealed a parallel increase in NPY-IR levels [208].

Maternal deprivation is an animal model of depression/vulnerability to stress that posits early life stress may cause changes in the CNS (e.g. hypothalamic-pituitary adrenal dysregulation) that are associated with an increased risk of adult life depressive psychopathology [209]. Using this model in rats for three hours per day during postnatal days 2-14, NPY levels were shown to be reduced in the hippocampus and striatum and increased in the hypothalamus [210]. However, if lithium treatment was employed on days 50-83, the changes in NPY-IR induced by maternal deprivation were not observed in the hippocampus and striatum while NPY levels were further increased in the hypothalamus [210]. Consequently, early life stress has long-term effects on NPY levels in the CNS and may be a factor in the development of depression; possibly through an increased vulnerability to stress.

4.2 Antidepressant-like effects of exogenous NPY

The Porsolt forced swim test is widely used for the screening of potential antidepressant drugs [211]. It has recently been shown that NPY displayed antidepressant-like activity in the rat forced swim test [212]. These results have since been confirmed in the mouse version of this test [213]. ICV NPY administration significantly reduced immobility time in a dose dependent manner, as did

[Leu^{31}Pro^{34}]PYY [213]. Attempts to determine the specific receptor subtype(s) involved has shown that BIBP3226 and BIBO3304 (selective Y_1 antagonists) and NPY(13-36) (a preferential Y_2 agonist) did not display any activity at the doses tested. However, pre-treatment with BIBP3226 and BIBO3304 significantly blocked the anti-immobility effects of NPY [213]. It is predicted that potentiation of NPY signaling at central postsynaptic receptors through Y_1 receptors is essential to reverse the behavioral characteristics of depression. The up-regulation of NPY expression through Y_1 receptor activation may be advantageous for behavioral adaptation to stress-induced changes in neuroplasticity. A maladaptive response to stress may be the result of an intrinsic abnormal function of central NPY transmission that is characteristic of depressive states.

4.3 Antidepressant-like effect by knocking out the Y_2 receptor

It was recently shown that Y_2 receptor knockout mice display antidepressant-like activity in the forced swim test [160]. The Y_2 deficient mice displayed approximately 3 times less immobility than wildtype mice indicating a stronger ability to cope with stress [160]. Additional studies with NPY knockout and transgenic animals, as well as the development of double and triple (Y_1,Y_2,Y_4) receptor knockout models [214] will greatly facilitate the attempt to understand the role of NPY in the psychopathology of depression.

4.4 Clinical support for the role of NPY in depression

The clinical data implicating a role for NPY in depressive disorder is somewhat limited. Several studies have demonstrated decreased NPY levels in the cerebrospinal fluid (CSF) [215;216] and platelet-poor plasma [217;218] of depressed patients, when compared to healthy control subjects. Interestingly, NPY-IR in the platelets of these depressed patients was significantly increased [218]. These results suggest that NPY release may be reduced, or that the metabolism of the peptide is increased, in the CNS of

depressed subjects [218]. Conversely, other studies involving patients suffering from major affective disorders failed to reveal any significant changes in CSF levels of NPY [219]. A negative correlation between CSF NPY and scores of anxiety in clinically depressed patients has been demonstrated [220], suggesting a possible link between low concentrations of NPY and predisposition to anxiety-related or stress-induced depression. Other studies have found decreases in NPY mRNA in the frontal cortex of patients with bipolar disorder using quantitative *in situ* hybridization [221] as well as DNA microarray [222]. A recent study has reported decreased CSF NPY in patients with treatment-refractory unipolar depression and presented preliminary evidence for a prepro-NPY gene polymorphism in these patients [223]. The authors predict that a Pro7/Leu7 substitution is associated with decreased NPY signaling through altered prepro-NPY processing [223]. The evidence from these clinical studies supports the theory that impaired NPY signaling may contribute to the manifestation of depression.

Despite the evidence for NPY in clinical depression, there are significant inconsistencies found in studies involving suicide and suicide attempts associated with depressive illness. Initial analysis revealed decreased NPY concentrations in the frontal cortex and caudate nucleus of suicide victims, which appeared to be particularly evident in subjects affected by major depression [224]. In contrast, studies published 3 years later demonstrated no significant differences in frontal cortex NPY levels between control subjects and subjects who were deemed affected by major depression [225]. A recent study examined NPY levels in 13 patients with a recent suicide attempt and the effect of antidepressant treatment in 7 of these patients. There was no significant difference in CSF NPY levels in the 6 patients who were not treated with antidepressants [226]. In the 7

patients who were given antidepressants, CSF (taken every 3 or 4 months after an initial wash-out period) NPY levels decreased between the 2nd and 3rd lumber puncture. There was also a trend toward a negative correlation between anxiety and CSF NPY levels at the time of puncture [226]. It is difficult to draw any conclusions based on this study because of the small sample size, co-morbid personality disorders, and the extreme heterogeneity of the patients. Additionally, these patients were without clinical improvement despite antidepressant treatment. It is unclear if NPY has a role in suicide and suicide attempts that are associated with major depression. Further studies are required to elucidate this possibility.

4.5 Prospective mechanisms for NPY in the pathophysiology of depression

There is evidence that lowered NPY levels may play a role in the pathogenesis of mood disorders and that one therapeutic mechanism of antidepressant drugs is to increase NPY levels. Chronic antidepressant treatment has been shown to increase NPY and NPY Y_1 receptor mRNA levels [195], and to reduce NPY Y_2 receptor densities in certain brain regions [227]. For example, chronic treatment with the tricyclic antidepressant imipramine, increased NPY-IR in the frontal cortex [228] and decreased [^{3}H]-NPY binding in frontal cortex and hippocampus of rats [227]. Sub-chronic treatment with imipramine was also shown to ameliorate the loss of NPY-positive interneurons in the hilus of the hippocampus caused by learned helplessness [229]. Similar results were obtained following treatment with lithium, an often employed pharmacotherapy for bipolar disorders [230]. Previously, it has been shown that lithium increased levels of prepro-NPY mRNA and NPY-IR in hippocampal and cortical regions [231]. In contrast, chronic treatment with the SSRI citalopram did not induce any significant changes in NPY-IR in rat hippocampal homogenates after chronic treatment [230;232]. Citalopram

treatment did, however, increase [^{125}I]-PYY binding sites in the hippocampus, changes representative of a possible increase in expression, or decreased degradation, of NPY-sensitive receptors [230]. Another possible mechanism may be related to the ability of citalopram to increase the affinity of NPY-sensitive receptors for the endogenous ligand [230] and hence increase NPY neurotransmission.

Additional experiments have shown that repeated, but not single, electroconvulsive shock stimulation in rats, the widely accepted animal model for electroconvulsive therapy in humans, increased NPY gene expression [233]. Such treatment also markedly increased levels of NPY-IR in homogenates of hippocampal and cortical regions [234], hilus of the dentate gyrus [233], and prepro-NPY mRNA in the stratum oriens of the hippocampus [235]. Electroconvulsive shock also significantly increased extracellular levels of NPY in the dorsal hippocampus of freely moving rats as determined by microdialysis, suggesting that such treatment led to an increased biosynthesis and release of NPY in this region [16;210]. Supporting this conclusion is the recent report that chronic, but not acute, electroconvulsive therapy led to a doubling of NPY mRNA transcription in the hippocampus and frontal cortex in rats as measured by high-density oligonucleotides microarray analysis [236]. Based on these studies, there is evidence that NPY may have a role in mood disorders such as depression.

Chapter 5: Objective One: Emotional behavior in NPY receptor knockout mice

The role of the NPY Y_1 and Y_2 receptor subtype has been examined using various pharmacological agents. In the rodent brain, the Y_1 receptor has predominant expression in the cerebral cortex, hippocampus, thalamus and brainstem nuclei [237]. The Y_2 receptor is abundantly expressed in the hippocampus and brainstem nuclei [237]. Based on these data, NPY produces robust anxiolytic-like and antidepressant-like activity that is mediated through the Y_1 (and possibly Y_5) receptor subtype while anxiogenic-like effects of NPY are primarily mediated through Y_2 receptors. However, mice deficient in the Y_2 receptor subtype display an anxiolytic-like phenotype in the elevated plus-maze and open field test [159;160] and express an antidepressant-like phenotype in the forced swim test [160]. Although the behavioral phenotype of NPY Y_2 receptor knockout mice and wildtype controls is known, the long term effect of the deletion of this receptor in aged animals is unknown. Therefore, anxiety and depression-related behaviors were investigated in aged two-year-old NPY Y_2 receptor knockout mice and wildtype controls. In addition, the behavioral phenotype of NPY Y_1 receptor knockout mouse in tests of anxiety and depression is unknown. Thus, the Y_1 knockout mice and Y_1 wildtype mice were also tested in the elevated plus-maze, open field test, and forced swim test.

5.1 Methodology

5.1.1 Animals

The generation of germline NPY Y_1 knockout mice is described in [23]. Using cre/loxP technology, a targeting vector for the Y_1 receptor gene was designed so that the entire coding region of the Y_1 receptor could be removed. NPY Y_1 knockout ($Y_1^{-/-}$ KO) mice and controls ($Y_1^{+/+}$ WT) are maintained on a mixed C57BL/6-129SvJ background and were received from Dr. Herbert Herzog at the Garvan Institute of Medical Research

in Sydney, Australia. Mice were subjected to behavioral tests when they were approximately 1 year old.

NPY Y_2 knockout ($Y_2^{-/-}$ KO) and NPY Y_2 wildtype ($Y_2^{+/+}$ WT) mice (C57/B16-129SvJ background) were developed using cre/loxP technology as previously described [238;239] and were also received from Dr. Herbert Herzog. These mice were tested at approximately 2 years of age. All animals were housed under standard laboratory conditions (12/12 h light/dark cycle, lights on at 07:00 h, food and water *ad libitum*). Animal care was provided according to protocols and guidelines approved by McGill University and the Canadian Council of Animal Care.

5.1.2 Elevated plus-maze

The test was performed as previously described [159;240-242]. The experimental apparatus consisted of a plus-formed maze elevated 50 cm above the ground. The four arms were 37.5 cm long and 5 cm wide. Two opposing arms were surrounded by black Plexiglas walls 15 cm high (closed walls); while the other arms were devoid of walls (open arms). The animal is placed in the center of the maze facing an open arm, after which the cumulative time spent in each arm and the number of entries into the open or closed arms were recorded during a 5 min test session. An individual entry into the arm is defined as the animal placing all four paws in that arm. The time spent in the open arms is expressed as a percentage of the total time spent in the arms (% time), and the number of entries in the open arms as a percentage of the total numbers of entries (% entry). The data is shown graphically as a percentage of the [open/(open + closed)] in both the number of entries and time spent in the open arms (% open) as well as # entries into the open arms and time spent on the open arms. Total number of entries onto any arm is

presented as a measure of general locomotor activity on the maze so as to rule out any non-specific effects that may have interfered with the interpretation of the data.

5.1.3 Open Field Test

The open field consists of a square base (70 cm X 70 cm) surrounded by a 75 cm high wall. Illumination is provided by a 40 W bulb, positioned 90 cm above the floor of the apparatus. The animals are placed into the center of the apparatus and the time spent in the central area of the arena is expressed as a percentage of the total time (% time). The number of crossings into the central area is expressed as a percentage of the total number of crossings (% entry). Total crossings are presented as a measure of general locomotor activity in the arena. Testing is conducted over a 10 min period and recorded by a video tracking system connected to a computer equipped with the commercially available HVS image system (HVS, UK) for the analysis of the open field activity.

5.1.4 Forced Swim Test

The Porsolt forced swim test is a reliable tool for screening potential antidepressant drugs [211]. In the mouse forced swim test, mice are individually placed in a 40cm-diameter cylinder filled with 24-25°C water to a depth of 30 cm for 6 minutes. Immobility time is recorded during the last 4 minutes. The immobility score is associated with a positive antidepressant-like effect.

5.1.5 Statistical Analysis

Behavioral data was analyzed with unpaired Student's t-tests using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). All results are reported as mean ± SEM. For all statistical analyses, $p < 0.05$ is considered statistically significant.

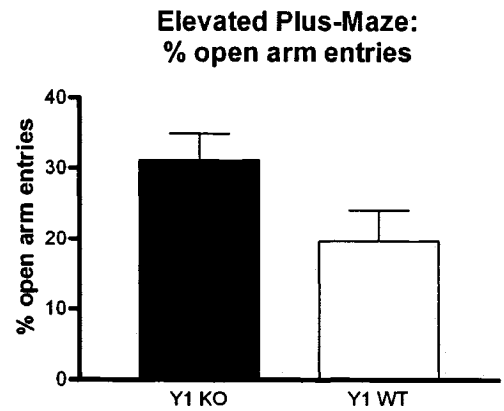
5.2 Results: Emotional behavior in NPY receptor knockout mice

Result 5.1: The behavioral profile of NPY $Y_1^{-/-}$ and $Y_1^{+/+}$ mice in the elevated plus-maze

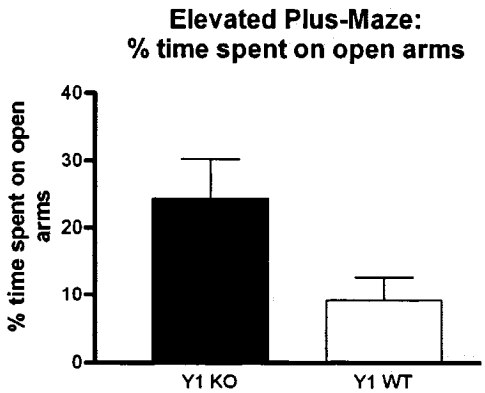
In the elevated plus-maze, the percent entries onto the open arms (Figure 5.1A, % Open Arm Entries; mean ± SEM: 31.14 ± 3.831 [KO] vs. 19.72 ± 4.358 [WT]; t=1.969; df=14; p = 0.0691) is not statistically significant between NPY Y_1 KO mice (n=9) and wildtype control mice (n=7). The percent time spent in the open arms (Figure 5.1B, % Time Spent on Open Arms; mean ± SEM: 24.36 ± 5.959 [KO] vs. 9.256 ± 3.405 [WT]; t=2.033; df=14; p = 0.0615) is not significant. There was no significant change in locomotor activity as the number of closed arm entries did not significantly differ between groups (Figure 5.1C, Total Crossings; mean ± SEM: 13.67 ± 1.633 [KO] vs. 15.00 ± 2.330 [WT]; t=0.4830; df=14; p = 0.6365).

Figure 5.1

5.1A



5.1B



5.1C

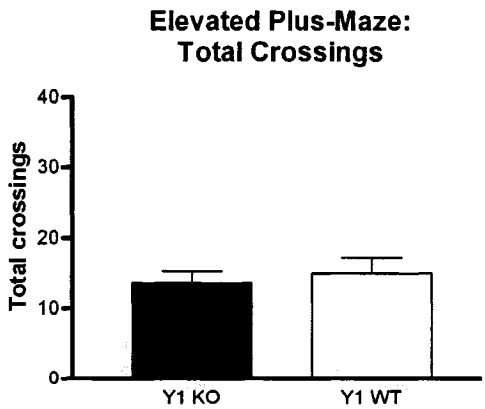
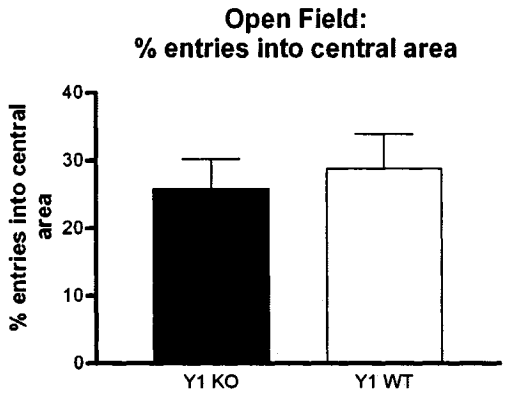


Figure 5.1: The behavioral profile of NPY $Y_1^{-/-}$ [Y1 KO] and $Y_1^{+/+}$ [Y1 WT] mice in the elevated plus-maze. Results are expressed as mean ± SEM of (A) % entries, (B) % time spent on open arms, and (C) total arm entries. Data analyzed using a two-tailed unpaired t-test.

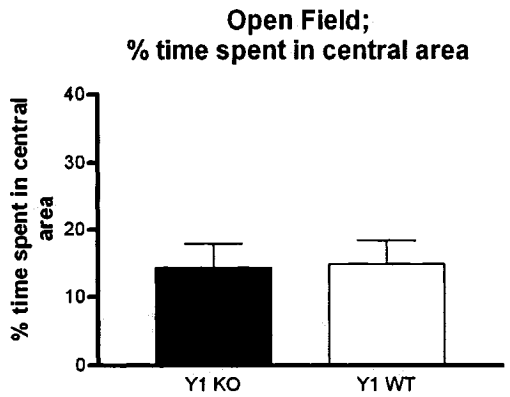
Result 5.2: The behavioral profile of NPY $Y_1^{-/-}$ and $Y_1^{+/+}$ mice in the open field

NPY Y_1 KO mice (n=8) did not display increased preference for the central area of the open field (Figure 5.2A; % Entries into Central Area; mean ± SEM: 25.85 ± 4.411 [KO] vs. 28.85 ± 5.133 [WT]; t=0.4456; df=13; p = 0.6632) and (Figure 5.2B; % Time Spent in Central Area; mean ± SEM: 14.46 ± 3.523 [KO] vs. 14.94 ± 3.468 [WT]; t=0.09659; df=13; p = 0.9245), when compared to wildtype animals (n=7). There was a significant change in locomotor activity in Y_1 KO mice (Figure 5.2C; Total Crossings; mean ± SEM: 272.6 ± 44.72 [KO] vs. 147.1 ± 28.17 [WT]; t=2.293; df=13; p = 0.0392), when compared to wildtype controls.

Figure 5.2
5.2A



5.2B



5.2C

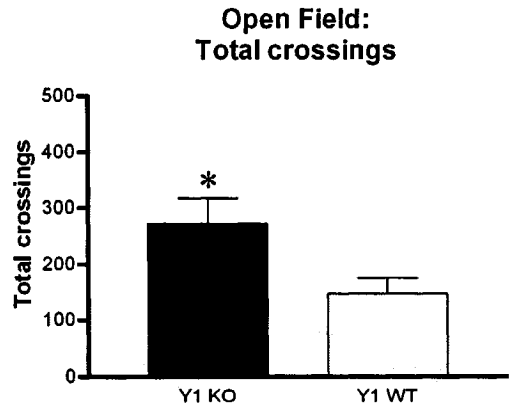


Figure 5.2: The behavioral profile of NPY $Y_1^{-/-}$ [Y1 KO] and $Y_1^{+/+}$ [Y1 WT] mice in the open field. Results are expressed as mean ± SEM of (A) % entries, (B) % time spent in central area, and (C) total arm entries. * $p < 0.05$ by two-tailed unpaired t-test.

Result 5.3: The behavioral profile of NPY $Y_1^{-/-}$ and $Y_1^{+/+}$ mice in the forced swim test

An unpaired t-test (two-tailed) did not reveal a significant difference in immobility scores between NPY Y_1 KO mice (n=7) and wildtype controls (n=7) (Figure 5.3, Immobility, Mean ± SEM: 14.29 ± 2.306 [KO] vs. 16.14 ± 1.71 [WT]; t=0.6468; df=12; p = 0.53) in the forced swim test.

Figure 5.3

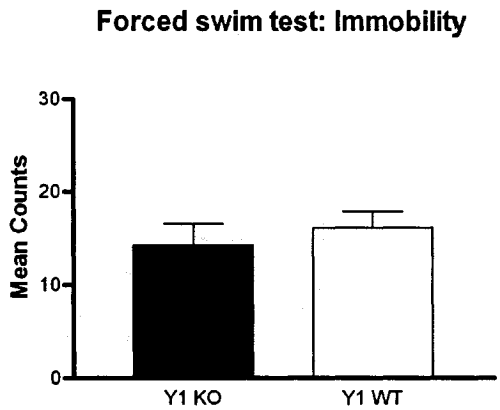


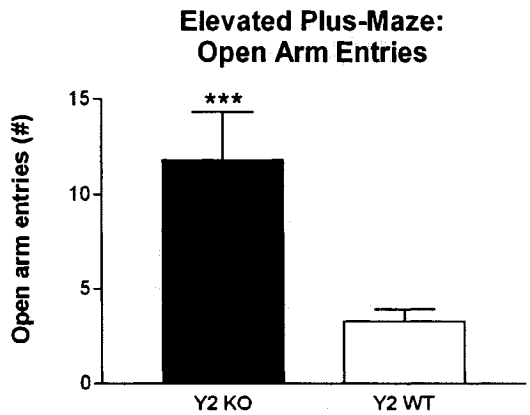
Figure 5.3: The behavioral profile of NPY $Y_1^{-/-}$ [Y1 KO] and $Y_1^{+/+}$ [Y1 WT] mice in the forced swim test. Results are expressed as mean ± SEM. Data analyzed by two-tailed unpaired t-test.

Result 5.4: The behavioral profile of aged NPY $Y_2^{-/-}$ and $Y_2^{+/+}$ mice in the elevated plus-maze

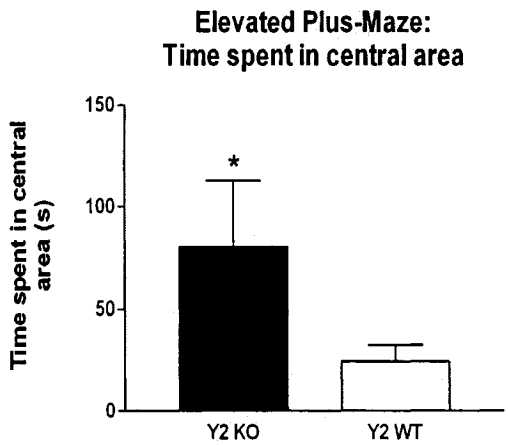
NPY Y_2 KO mice (n=6) made more entries (Figure 5.4A, Open Arm Entries; mean ± SEM: 11.83 ± 2.469 [KO] vs. 3.250 ± 0.6643 [WT]; t=4.422; df=16; p = 0.0004) and spent significantly more time (Figure 5.4B, Time Spent in Central Area; mean ± SEM: 80.50 ± 32.30 [KO] vs. 24.33 ± 8.059 [WT]; t=2.250; df=16; p = 0.0389) on the open arms of the elevated plus-maze compared to wildtype control mice (n=12). The percent entries onto the open arms (Figure 5.4C, % Open Arm Entries; mean ± SEM: 35.34 ± 7.493 [KO] vs. 21.94 ± 3.292 [WT]; t=1.921; df=16; p = 0.0728) is not statistically significant while the percent time spent in the open arms (Figure 5.4D, % Time Spent on Open Arms; mean ± SEM: 29.81 ± 10.39 [KO] vs. 9.705 ± 3.165 [WT]; t=2.382; df=16; p = 0.0300) is significant. This effect was not due to non-specific changes in locomotor activity as the number of closed arm entries did not significantly differ between groups (Figure 5.4E, Total Crossings; mean ± SEM: 27.67 ± 4.709 [KO] vs. 16.83 ± 2.878 [WT]; t=2.066; df=16; p = 0.0554).

Figure 5.4

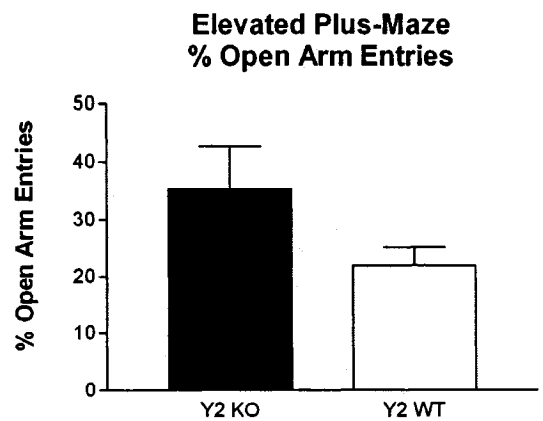
5.4A



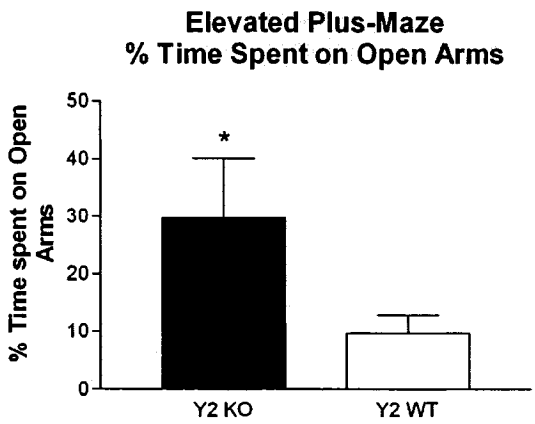
5.4B



5.4C



5.4D



5.4E

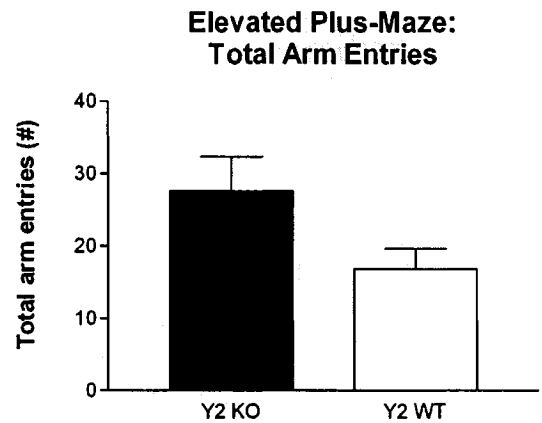


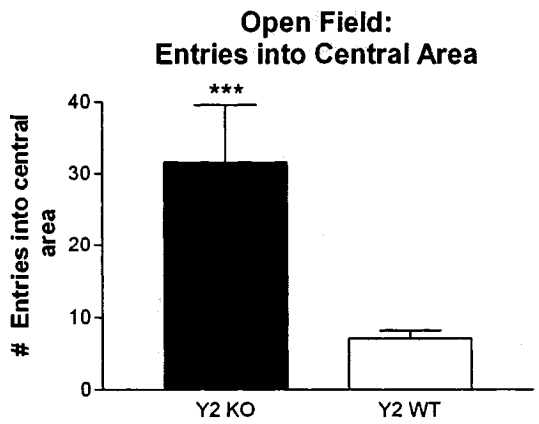
Figure 5.4: Behavioral profile of aged NPY $Y_2^{-/-}$ [Y2 KO] and $Y_2^{+/+}$ [Y2 WT] mice in the elevated plus-maze. Results are expressed as mean ± SEM of (A) entries, (B) time spent on open arms, (C) % entries, (D) % time spent on open arms, and (E) total arm entries. * $p < 0.05$ and *** $p < 0.0001$ by two-tailed unpaired t-test.

Result 5.5: The behavioral profile of aged NPY $Y_2^{-/-}$ and $Y_2^{+/+}$ mice in the open field

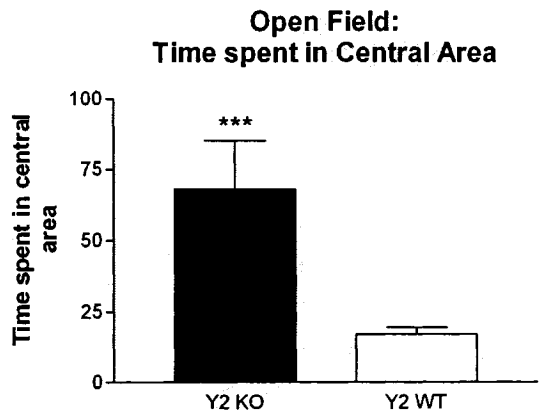
NPY Y_2 KO mice (n=5) displayed increased preference for the central area of the open field (Figure 5.5A; Entries into Central Area; mean ± SEM: 31.60 ± 8.016 [KO] vs. 7.083 ± 1.041 [WT]; t=4.720; df=15; p = 0.0003) and (Figure 5.5B; Time Spent in Central Area; mean ± SEM: 68.20 ± 17.04 [KO] vs. 17.25 ± 2.346 [WT]; t=4.587; df=15; p = 0.0004), when compared to wildtype animals (n=12). However, the percent entries (Figure 5.5C; % Entries into Central Area; mean ± SEM: 9.118 ± 2.218 [KO] vs. 7.451 ± 1.680 [WT]; t=4.595; df=15; p = 0.5844) was not significant between groups. There was a significant difference between groups in the percent time spent in the central area (Figure 5.5D; % Time Spent in Central Area; mean ± SEM: 11.36 ± 2.841 [KO] vs. 2.841 ± 0.3955 [WT]; t=4.595; df=15; p = 0.0004). There was also a significant change in locomotor activity in Y_2 KO mice (Figure 5.5E; Total Crossings; mean ± SEM: 310.4 ± 50.59 [KO] vs. 117.4 ± 13.83 [WT]; t=5.079; df=15; p = 0.0001), when compared to wildtype controls.

Figure 5.5

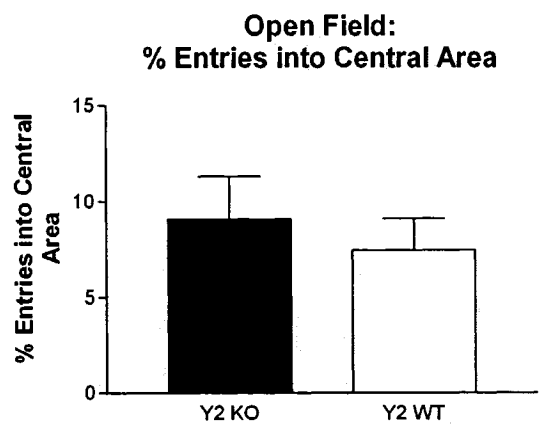
5.5A



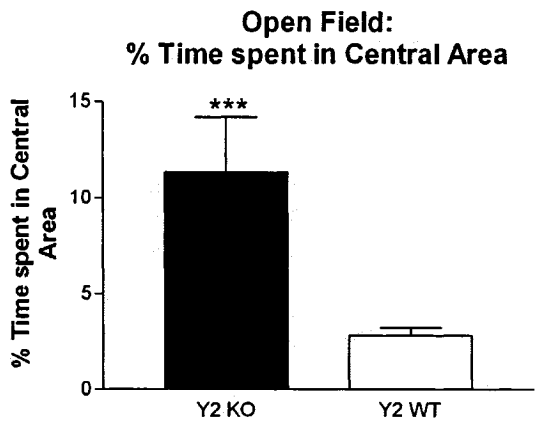
5.5B



5.5C



5.5D



5.5E

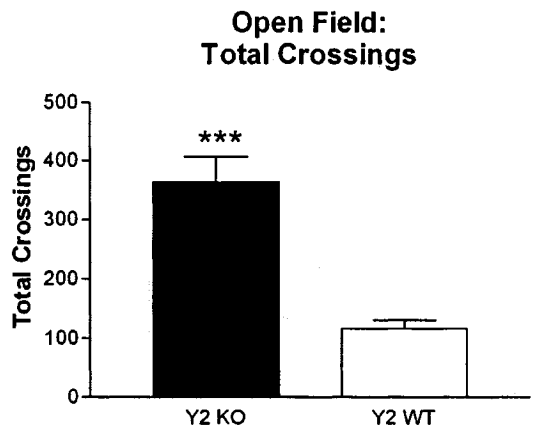


Figure 5.5: Behavioral profile of aged NPY $Y_2^{-/-}$ [Y2 KO] and $Y_2^{+/+}$ [Y2 WT] mice in the open field. Results are expressed as mean ± SEM of (A) entries, (B) time spent in central area, (C) % entries, (D) % time spent in central area, and (C) total crossings. * $p < 0.05$ and *** $p < 0.0001$ by two-tailed unpaired t-test.

Result 5.6: The behavioral profile of aged NPY $Y_2^{-/-}$ and $Y_2^{+/+}$ mice in the forced swim test

An unpaired t-test (two-tailed) revealed a significant difference in mean immobility scores between NPY Y_2 KO mice (n=5) and wildtype controls (n=12) (Figure 5.6, Immobility, Mean ± SEM: 4.400 ± 2.619 [KO] vs. 24.83 ± 3.593 [WT]; t=3.465; df=15; p = 0.0035) in the forced swim test.

Figure 5.6

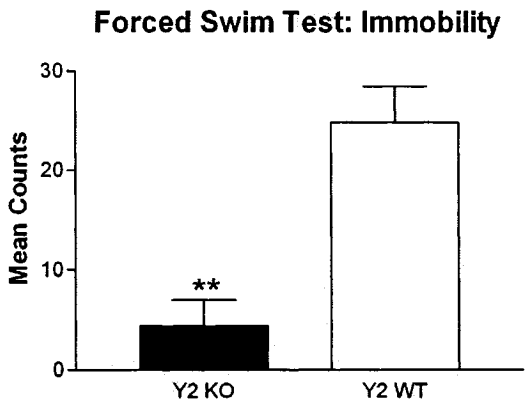


Figure 5.6: Behavioral profile of aged NPY $Y_2^{-/-}$ [Y2 KO] and $Y_2^{+/+}$ [Y2 WT] mice in the forced swim test. Results are expressed as mean ± SEM. ** $p < 0.02$ by two-tailed unpaired t-test.

5.3 Discussion

There was no significant difference in emotional behavior between $Y_1^{-/-}$ and $Y_1^{+/+}$ mice in the elevated-plus maze, open field test or forced swim test. There was a significant increase in locomotor activity in the open field test. This result is in agreement with one study [243] but not another [244]. It has been proposed that the discrepancies found between the pharmacological studies and the knockout results are most likely due to redundancies in the NPY system that lead to compensation during development [214]. Furthermore, a substantial increase in the number of α2-adrenoceptors in the locus coeruleus has been found in Y_1 receptor knockout mice compared to control animals [243]. The Y_1 receptors normally function to provide presynaptic noradrenergic inhibition so the deletion of these receptors may affect noradrenaline release. Interestingly, infusion of α_2-adrenoceptor antagonists and agonists into the locus coeruleus of rats can increase or decrease, respectively, activity in the forced swim test [245;246]. The α_{2A} and α_{2C}–adrenoceptor subtype levels are elevated both in the locus coeruleus of depressed patients [247] and by long-term stress in rats [248;248]. However, knockout studies suggest that mice lacking α_{2C}-adrenoceptors, but not α_{2A}-adrenoceptors, show an antidepressant profile [249]. In fact, the lack of α_{2A}-adrenoceptors elicits a depressive response in the forced swim test and anxiety [250]. Although it is unknown which subtype of the α_2-adrenoceptor was increased in the locus coeruleus of Y_1 knockout mice compared to wildtype controls, this developmental compensation may be one factor that influenced the behavior observed in these mice.

Another possibility is that the predicted anxiogenic-like phenotype of NPY Y_1 KO mice was unable to be deteted using only the elevated plus-maze and open field test. The limitation of using these tests is that they are restricted to the approach/avoidance

category of anxiety tests that are based on the conflict between the natural aversion of rodents for open spaces and the drive to explore a novel environment. The other categories include punished responding, conditioned fear, and aggression/social behavior-based tests. This theory is supported by a study that found a significantly higher territorial aggression in Y_1 knockout mice compared to control animals using established aggression paradigms [243].

It has previously been shown that mice deficient in the Y_2 receptor subtype have an anxiolytic-like phenotype in the elevated plus-maze and open field test without changes in locomotor activity [159]. These findings have been confirmed by an independent laboratory using the open field, elevated plus-maze, and light-dark compartment test [160]. An increase in locomotor activity was observed in the open field when mice were tested with light, but not when they were tested in the dark [160]. It was also shown that $Y_2^{-/-}$ mice displayed approximately 3 times less immobility in the forced swim test than wildtype controls indicating a stronger ability to cope with stress [160]. The anxiolytic-like profile of the Y_2 deficient mouse was confirmed in two-year old Y_2 knockout mice. An increase in locomotor activity in the open field was also observed in the open field test; most likely because these mice were tested with light. The interaction between CRF and NPY has been proposed as a means in which emotional behavior is regulated in NPY Y_2 receptor knockout mice. For example, deletion of Y_2 receptors causes a 60% reduction in CRF mRNA expression [178] that might contribute to the anxiolytic-like behavior of NPY Y_2 receptor knockout mice [160]. These data provide further evidence that modulators of the NPY Y_2 receptor subtype are potential drug targets for the treatment of anxiety disorders in human subjects.

Chapter 6: Objective Two: Cell Proliferation in NPY Y_2 receptor KO mice

There is evidence that NPY is involved in neuroproliferation in the CNS. For example, in the olfactory bulb, NPY functions to promote neuroproliferation in postnatal precursor cells [22]. NPY Y_1 deficient mice developed significantly fewer olfactory neurons by adulthood. NPY $Y_1^{-/-}$ mice contained half the number of dividing neuronal precursors than control animals suggesting that the Y_1 receptor mediates early steps in olfactory neurogenesis by increasing the number of basal cells undergoing cell division [22]. NPY was also recently characterized as a neuroproliferative factor mediated by Y_1 receptors in primary cultures from the SGZ of the dentate gyrus [23]. The greatest concentration of hippocampal Y_1 receptors are found in the molecular, granule, and SGZ of the dentate gyrus, supporting NPY as a potential modulator of hippocampal neurogenesis [23]. Interestingly, a significant decrease in dentate gyrus proliferation in NPY Y_1 receptor knockout mice compared to wildtype control animals was found [251]. However, the total number of neurons in the granule cell layer was unchanged between groups [251]. This suggests that Y_1 KO mice compensate for the decrease in hippocampal proliferation; possibly by increasing survival of newly born neurons in the dentate gyrus. This may explain why there was no difference in immobility scores in the forced swim test in NPY Y_1 KO mice compared to wildtype controls. There is, however, a significant antidepressant-like effect in the forced swim test in NPY $Y_2^{-/-}$ mice compared to $Y_2^{+/+}$ mice. Y_2 receptors are considered to be autoreceptors that provide negative feedback to NPY-ergic nerve terminals to modulate NPY release [89]. NPY $Y_2^{-/-}$ mice are predicted to have increased endogenous peptide expression that may act on Y_1 receptors to modulate behavior and neuroproliferation. Thus, proliferation was examined in NPY $Y_2^{-/-}$ and $Y_2^{+/+}$ mice in the dentate gyrus of the hippocampus and SVZ.

6.1 Methodology
Neurogenesis (Proliferation)
6.1.1 Bromodeoxyuridine injections

Bromodeoxyuridine (BrdU) (Sigma, St Louis, MO, USA) was prepared in saline to a dilution of 20 mg/mL BrdU with 0.007M NaOH. The solution was dissolved by sonication. In order to examine proliferation of precursor neurons, animals were given a single intraperitoneal injection of BrdU (200 mg/kg) with a survival time of 2 hours. A survival time of 2 hours ensures that cells in the synthesis phase of mitosis incorporate the thymidine analogue BrdU, but do not complete mitosis [252].

6.1.2 Perfusion and tissue storage:

Animals were given an overdose of ketamine/xylazine and transcardially perfused first with phosphate buffered saline (PBS) and subsequently with 4% paraformaldehyde in 0.1M phosphate buffer (PB pH 7.4) according to protocols and guidelines approved by McGill University and the Canadian Council of Animal Care. Serial 40μm-thick sections throughout the entire SVZ and dentate gyrus (DG) were taken using a cryostat and stored in cryoprotectant (25% ethylene glycol, 25% glycerol, 0.05M PB, pH 7.4) at -20°C.

6.1.3 BrdU Immunohistochemistry

To examine the proliferation of precursor cells, the protocol of Pham et al., [78] was followed. Every 6th section from each brain (n=5 [Y_2 KO] n=6 [Y_2 WT]) was processed for BrdU immunohistochemistry. Sections were treated with 0.6% hydrogen peroxide in 0.1 M TBS (0.15M NaCl, 0.1M Tris-HCl, pH 7.5) for 30 min to block endogenous peroxidase. For DNA denaturation, the sections were incubated in 50% formamide/2X SSC (0.3M NaCl and 0.3M sodium citrate) at 65°C, rinsed for 5 min in 2X SSC, incubated in 2N HCl for 30 min at 37 °C, and then placed in 0.1M boric acid (pH 8.5) for 10 min. Following several rinses in TBS, slices were incubated in TBS++ (TBS; 0.1%

Triton-X100; 3% normal goat serum) for 30 min and incubated overnight at 4 °C with rat anti-BrdU primary antibody (1:200 in TBS++). The following day, the sections were rinsed in TBS and incubated in biotinylated goat anti-rat secondary antibody (1:200 in TBS++) for 1 hour at room temperature. Following intermittent rinses in TBS, avidin–biotin–horseradish peroxidase (ABC kit) was applied for 1 hour, followed by peroxidase detection with 3,3′-diaminobenzidine (DAB). The sections were dehydrated in a series of ethanol, cleared in xylene, and coverslipped with DEPEX mounting medium.

6.1.4 Quantification of BrdU labeling

In order to determine the total number of proliferating cells in the DG and SVZ, every 6^{th} section from each animal was viewed on a Nikon E800 microscope. This spacing ensures that the same cell is not counted in more than one section. BrdU-labeled cells were identified and counted at 400x and 1000x magnification to distinguish single cells within clusters, omitting cells appearing in the upper focal plane [78]. A BrdU-positive cell was counted as being in the SGZ of the dentate gyrus if it was touching or within a two cell distance from the SGZ. Cells that were located more than two neurons away from the SGZ were classified as hilar. BrdU-labeled cells were counted in the entire extent of the DG (SGZ, granule cell layer and hilus combined).The total number of BrdU-positive cells per section was determined and multiplied by six to obtain the total number of cells per DG. The number of BrdU-positive cells on the lateral side of the lateral ventricle was considered a measure of SVZ proliferation.

6.1.5 Statistical Analysis

Cell counts were analyzed with unpaired Students's t-tests using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). All results are reported as mean ± SEM. For all statistical analyses, $p < 0.05$ is considered statistically significant.

6.2 Results

Result 6.1: Cell Proliferation in Y_2 KO and Y_2 WT Mice

6.1A: Cell Proliferation in the Dentate Gyrus of Y_2 KO and Y_2 WT Mice

An unpaired t-test (two-tailed) did not reveal a significant difference between the number of BrdU-positive cells in the dentate gyrus of NPY Y_2 KO mice and wildtype controls (Figure 6.1A, Dentate Gyrus Cell Proliferation, mean ± SEM: 81.60 ± 61.35 [KO] vs. 46.00 ± 10.81 [WT]; t = 0.6284; df = 9; p = 0.5454).

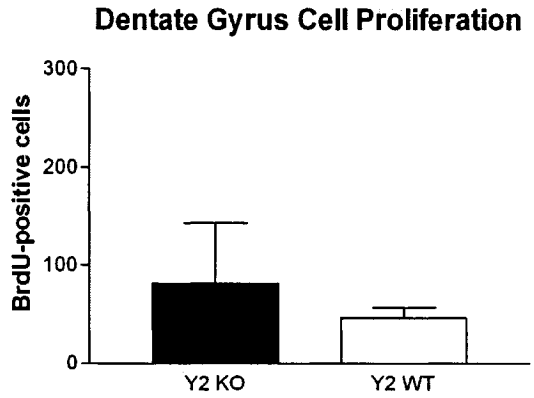
6.1B: Cell Proliferation in the Subventricular Zone of Y_2 KO and Y_2 WT Mice

An unpaired t-test (two-tailed) did not reveal a significant difference between the number of BrdU-positive cells in the SVZ of NPY Y_2 KO mice and wildtype controls (Figure 6.1B, SVZ Cell Proliferation, mean ± SEM: 1283 ± 173.0 [KO] vs. 1519 ± 336.5 [WT]; t=0.5855; df=9; p = 0.5726).

The proliferation of newly born cells in the dentate gyrus and subventricular zone was determined using BrdU immunohistochemistry and visualized with DAB staining (Figure 6.2 and Figure 6.3). BrdU-positive cells (brown) are present in the dentate gyrus 2 hours after BrdU administration in NPY Y_2 receptor knockout mice (6.2A) and Y_2 wildtype control mice (6.2B). BrdU-positive cells (brown) are also present in the subventricular zone 2 hours after BrdU administration in NPY Y_2 receptor knockout mice (6.3A) and Y_2 wildtype control mice (6.3B).

Figure 6.1

6.1A



6.1B

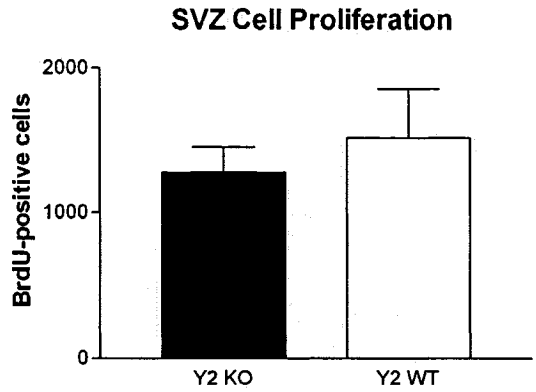
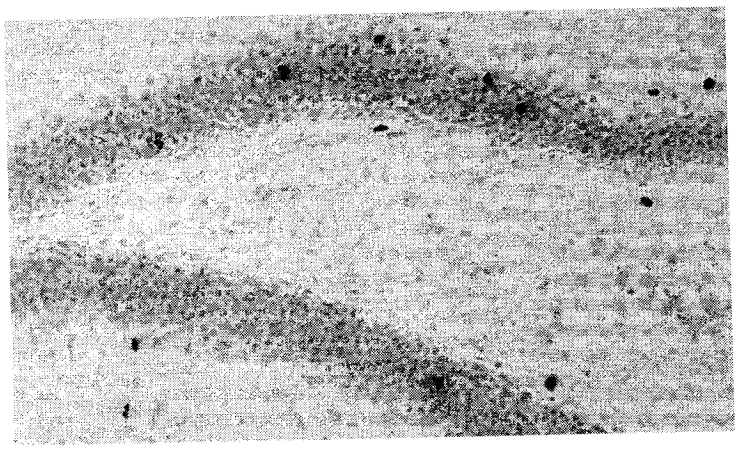


Figure 6.1: Proliferation in NPY $Y_2^{-/-}$ [Y2 KO] and $Y_2^{+/+}$ [Y2 WT] mice represented as the number of BrdU-positive cells in the dentate gyrus (6.1A) and SVZ (6.1B). Data are presented as mean ± SEM. Data analyzed with two-tailed unpaired t-test.

Figure 6.2

6.2A: Dentate Gyrus Cell Proliferation in Y_2 KO mice



6.2B: Dentate Gyrus Cell Proliferation in Y_2 WT mice

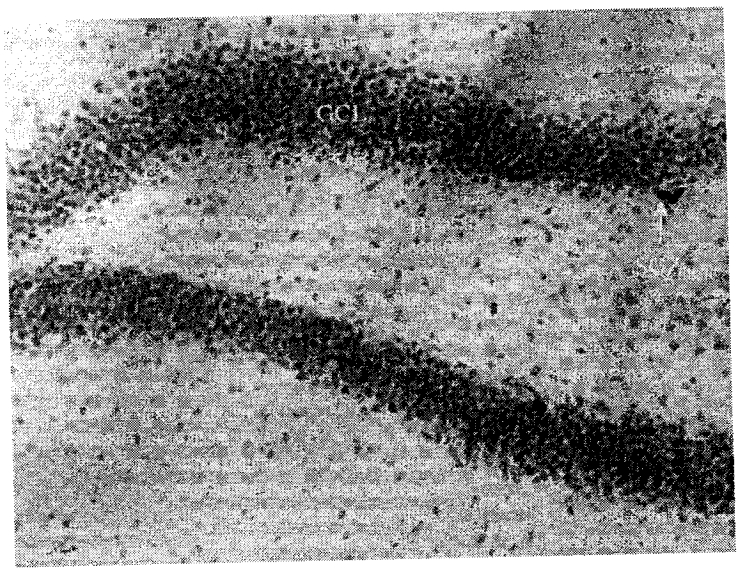
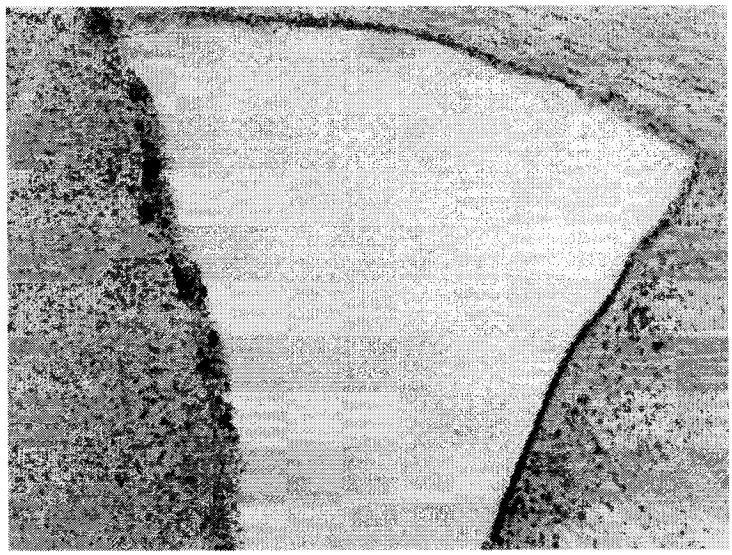


Figure 6.2: Representative photomicrographs (20x magnification) of BrdU-positive cells in the dentate gyrus of the hippocampus in NPY $Y_2^{-/-}$ mice (6.2A) and $Y_2^{+/+}$ mice (6.2B) visualized by DAB staining. The majority of the BrdU-labeled cells are located in the subgranular zone (SGZ), indicated by an *arrow* in 6.2B, the region between the granule cell layer (GCL) and hilus (H).

Figure 6.3

6.3A: SVZ Cell Proliferation in Y_2 KO mice



6.3B: SVZ Cell Proliferation in Y_2 WT mice

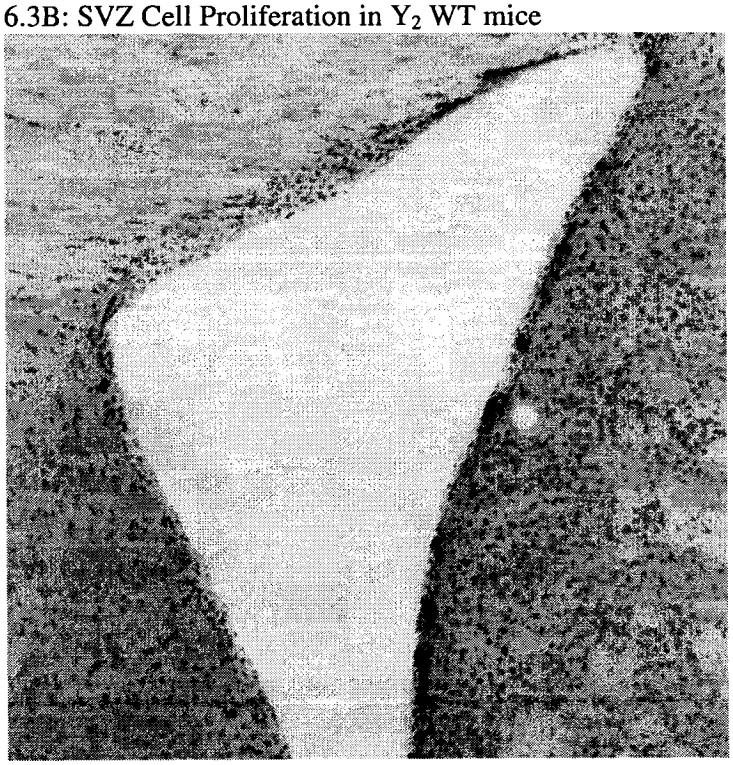


Figure 6.3: BrdU-positive cells (10x magnification) in the subventricular zone (SVZ) of NPY $Y_2^{-/-}$ mice (6.3A) and $Y_2^{+/+}$ mice (6.3B) visualized by DAB staining.

6.3 Discussion

There was no significant difference between the number of BrdU-positive cells in the DG or SVZ of NPY Y_2 KO and control mice in this study. There was a trend towards more BrdU-positive cells in the dentate gyrus of Y_2 KO mice compared to control mice despite the high degree of variability. The result for DG (but not SVZ) proliferation is unexpected as there was a significant difference in immobility scores between groups in the forced swim test. However, there is evidence that the rate of neurogenesis can be regulated by several factors. For example, increasing age is a factor that is known to decrease neurogenesis. A significant decrease (90%) in the number of BrdU-positive cells in the DG of aging rats [253] and mice [254] has been found. A reduction in the number of BrdU-positive cells has not been observed in the SVZ of aging rats [253] although there are conflicting results [255]. In mice, a significant reduction (60%) in the number of BrdU-positive neurons has been found in the SVZ [256]. The NPY Y_2 KO and control mice used in this study are considered to be aged or "senescent" mice because they are older than 20 months [257]. Consequently, age was a confounding factor in these mice that most likely interfered with the interpretation of these results. The overall low level of proliferation in the SGZ might have overshadowed any differences between groups.

It has been suggested that decreased levels of proliferation in aged animals may be related to elevated levels of circulating glucocorticoids [258] or decreased levels of activating factors such as IGF-1 [259]. Future studies will need to explore this possibility in Y_2 KO and control mice. Proliferation in NPY Y_2 KO and control mice should also be examined in younger mice. The survival and differentiation of newly born neurons in the hippocampus should be determined in young as well as aged NPY Y_2 KO and wildtype control mice.

Chapter 7: Objective Three: Effect of chronic NPY treatment on emotional behavior and neurogenesis: Cell Proliferation

Preclinical and clinical data validates NPY as a potential therapeutic target for the treatment of depressive disorders. For example, NPY has an acute effect on antidepressant-like behavior in both the rat and mouse the forced swim test [212;213] and NPY was recently implicated as an inducer of neuronal precursor proliferation in the dentate gyrus of the hippocampus [251]. Given the increasing evidence that a wide range of antidepressant drugs stimulate hippocampal proliferation in animal models with chronic treatment [40], the ability of NPY to induce hippocampal proliferation after chronic treatment in a rat animal model should determine the possible efficacy of NPY as a potential antidepressant therapy.

The aim of this objective was to assess antidepressant-like behavior in the rat forced swim test and examine the level of hippocampal proliferation that occurred after chronic treatment with NPY. The Alzet® mini pump system was used to administer NPY peptide, saline (a negative control) or the traditional antidepressant fluoxetine (a positive control) for 14-days. During the last two days of treatment, behavior was measured in all groups. Antidepressant-like behavior was measured in the rat forced swim test and locomotor activity was measured in the open field test to rule out any non-specific effects of drug treatment. Proliferation in the dentate gyrus and the SVZ that occurred after a 14-day chronic treatment with NPY, saline, or fluoxetine was then examined. It is predicted that chronic treatment with NPY peptide and fluoxetine will increase hippocampal but not SVZ proliferation and have significant antidepressant-like effects in the forced swim test compared to saline-treated animals.

7.1 Methodology

7.1.1 Animals

Male Sprague-Dawley rats were housed under standard laboratory conditions (12/12 h light/dark cycle, lights on at 07:00 h, food and water *ad libitum*). Animal care was provided according to protocols and guidelines approved by McGill University and the Canadian Council of Animal Care.

7.1.2 Drug Treatment

Drug treatment was provided with the Alzet® mini osmotic pump and brain infusion system for 14 days. NPY was dissolved in saline containing 0.01% ascorbic acid and 0.2% BSA at a concentration that infused a total of 28μg NPY during a 14-day chronic treatment. The osmotic pumps (Alzet Model 2002) containing NPY peptide (n=8) were implanted subcutaneously, attached to a brain infusion system (Alzet Brain Infusion System II), implanted in the right lateral ventricle (posterior: 1.0 mm, ventral: 5.0 mm, lateral 1.5 mm), and secured with cyanoacrylate adhesive. Sterile saline was administered to a separate set of animals (n=13) via subcutaneous osmotic pump (Alzet Model 2002) or via subcutaneous osmotic pump (Alzet Model 2002) attached to a brain infusion system (Alzet Brain infusion system II). Fluoxetine (1mg/kg, dissolved in 25% saline and 75% polyethylene glycol) was administered to a separate set of animals (n =7) via subcutaneous osmotic pump (Alzet® Model 2ML2).

7.1.3 Forced Swim Test

In the rat forced swim test, animals are placed in a 19 cm-diameter cylinder filled with 22°C water to a depth of 28 cm for a 15 min pre-swim. The next day, rats are placed back into the water for 5 min, and their behavior is recorded every 5 s. Behavior is divided into five categories: immobility (forepaws immobile), passive swimming (forepaws moving

underwater), active swimming (forepaws breaking the water's surface), wall climbing, and diving. A lower immobility score is associated with positive antidepressant-like effects. The pre-swim was conducted on day 13 of drug treatment and testing was conducted on day 14 of drug treatment.

7.1.4 Open Field Test

Total crossings are presented as a measure of general locomotor activity in the open field. Testing is conducted over a 10 min period and recorded by a video tracking system connected to a computer equipped with the commercially available HVS image system (HVS, UK) for the analysis of the open field activity. Open field activity was recorded on day 13 of drug treatment.

7.1.5 Neurogenesis: Cell Proliferation

BrdU injections were given the day after drug treatment (day 15). Two hours after BrdU injection, animals were sacrificed and transcardially perfused as previously described in *Section 6.1.* BrdU immunohistochemistry and quantification of BrdU labeling was performed as previously described in *Section 6.1.* Sections were taken from each animal (n=5 [NPY], n=6 [saline], n=7 [Fluoxetine]) for dentate gyrus and (n=6 [NPY], n=6 [saline], n=7 [Fluoxetine]) for SVZ proliferation.

7.1.6 Statistical Analysis

Behavioral data and cell counts were analyzed with one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc tests of significance using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). All results are reported as mean ± SEM. For all statistical analyses, $p < 0.05$ is considered statistically significant.

7.2 Results: Effect of chronic NPY treatment on emotional behavior and neurogenesis: Cell Proliferation

Result 7.1: Forced Swim Test in NPY, saline, and fluoxetine-treated rats

Immobility

A between subjects analysis of variance (ANOVA) revealed a significant difference in immobility scores between groups during the forced swim test (Figure 7.1, Immobility; mean ± SEM: 14.63 ± 2.878 [NPY] vs. 23.77 ± 1.776 [Saline] vs. 15.71 ± 1.782 [Fluoxetine]; $F[2,25] = 6.009$, $p = 0.0074$). A Tukey's multiple comparison post hoc test revealed significant differences between NPY and saline ($p < 0.05$) and Fluoxetine and saline ($p < 0.05$) but not between NPY and Fluoxetine ($p > 0.05$).

Passive Swimming

A between subjects analysis of variance (ANOVA) did not reveal a significant difference in passive swimming between groups during the forced swim test (Figure 7.1, Passive Swimming; mean ± SEM: 26.38 ± 4.179 [NPY] vs. 19.62 ± 2.563 [Saline] vs. 23.29 ± 1.948 [Fluoxetine]; $F[2,25]= 1.344$, $p = 0.2789$).

Active swimming/Wall climbing

A between subjects analysis of variance (ANOVA) did not reveal a significant difference in active swimming/wall climbing between groups during the forced swim test (Figure 7.1, Active Swimming/Wall Climbing; mean ± SEM: 9.375 ± 2.039 [NPY] vs. 7.462 ± 0.9269 [Saline] vs. 10.79 ± 2.736 [Fluoxetine]; $F[2,53]= 0.9714$, $p = 0.3852$).

Diving

A between subjects analysis of variance (ANOVA) did not reveal a significant difference in diving between groups during the forced swim test (Figure 7.1, Diving; mean ± SEM: 1.250 ± 1.114 [NPY] vs. 2.154 ± 0.7147 [Saline] vs. 0.4286 ± 0.4286 [Fluoxetine]; $F[2,25]= 1.120$, $p = 0.3421$).

Figure 7.1

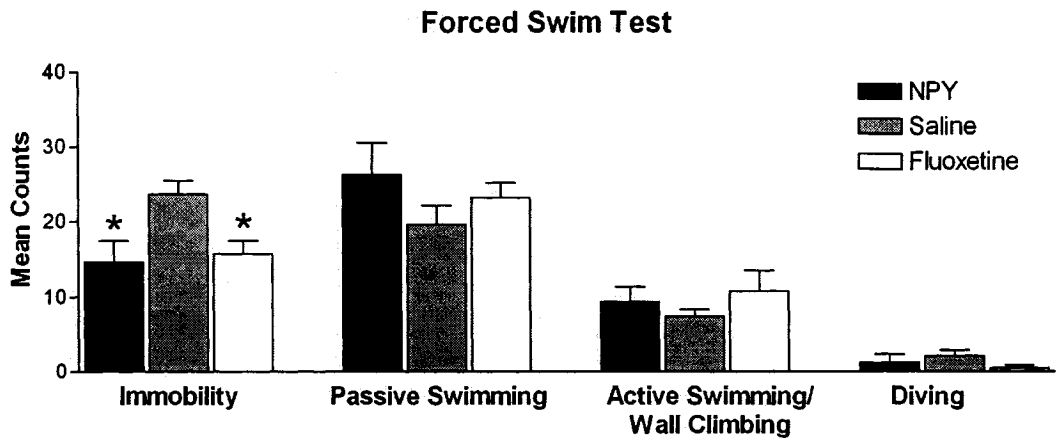


Figure 7.1: Behavior profile in the rat forced swim test after chronic treatment with NPY, saline, or fluoxetine. Behavior is recorded every 5 sec for 5 min. A lower immobility score is associated with positive antidepressant-like effects. Data are presented as mean ± SEM. Significant differences from saline. * $p < 0.05$ using between subjects ANOVA

Result 7.2: Open field test in NPY, saline, and fluoxetine-treated rats

A between subjects analysis of variance (ANOVA) did not reveal a significant difference in total crossings between groups during the open field test (Figure 7.2, Total Crossings; mean ± SEM: 195.8 ± 22.87 [NPY] vs. 178.1 ± 16.49 [Saline] vs. 156.1 ± 20.81 [Fluoxetine]; $F[2, 25]= 0.8150$, $p = 0.4540$).

Figure 7.2

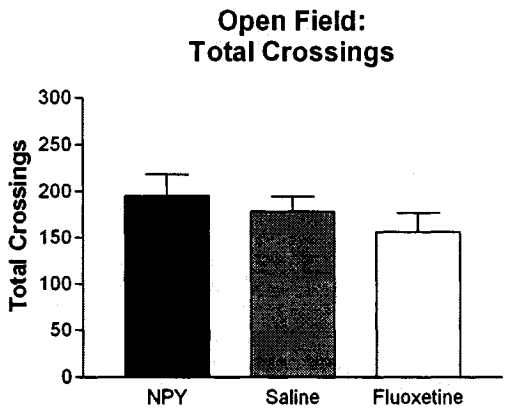


Figure 7.2: Locomotor activity in the open field test after chronic treatment with NPY, saline, or fluoxetine. Total crossings were recorded for 10 min. Data are presented as mean ± SEM. Data analyzed using between subjects ANOVA

Result 7.3A: Dentate Gyrus cell proliferation in NPY, saline, and fluoxetine-treated rats

A between subjects analysis of variance (ANOVA) revealed a significant difference between number of BrdU-positive cells in the dentate gyrus (Figure 7.3A, Dentate Gyrus Cell Proliferation; mean ± SEM: 732.0 ± 156.5 [NPY] vs. 470.0 ± 83.91 [Saline] vs. 1219 ± 271.1 [Fluoxetine]; $F[2,16] = 3.756$, $p = 0.046$). A Tukey's multiple comparison post hoc test revealed significant differences between Fluoxetine and saline ($p < 0.05$) but not between NPY and Fluoxetine ($p > 0.05$) or NPY and saline ($p > 0.05$).

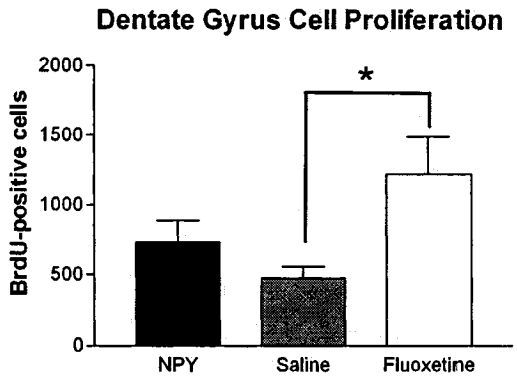
Result 7.3B: Subventricular Zone cell proliferation in NPY, saline, and fluoxetine-treated rats

A between subjects analysis of variance (ANOVA) did not reveal a significant difference in the number of BrdU-positive cells in the subventricular zone between groups (Figure 7.3B, SVZ Cell Proliferation; mean ± SEM: 2221 ± 388.6 [NPY] vs. 1373 ± 199.5 [Saline] vs. 1552 ± 261.0 [Fluoxetine]; $F[2,15]= 2.271$, $p = 0.1376$).

The proliferation of newly born cells in the dentate gyrus and subventricular zone was determined using BrdU immunohistochemistry and visualized with DAB staining (Figure 7.4 and Figure 7.5). BrdU-positive cells (brown) are present in the dentate gyrus 2 hours after BrdU administration following a 14 day chronic treatment with NPY (7.4A), saline (7.4B) or fluoxetine (7.4C). BrdU-positive cells (brown) are also present in the subventricular zone 2 hours after BrdU administration following a 14 day chronic treatment with NPY (7.5A), saline (7.5B) or fluoxetine (7.5C).

Figure 7.3

7.3A



7.3B

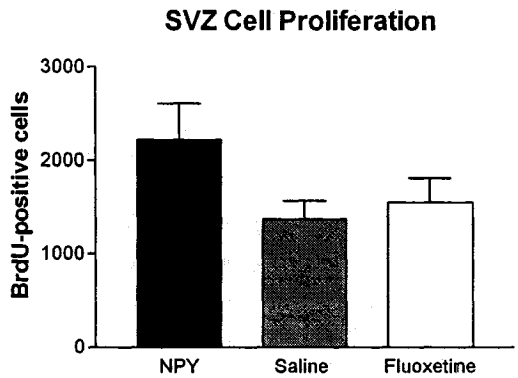
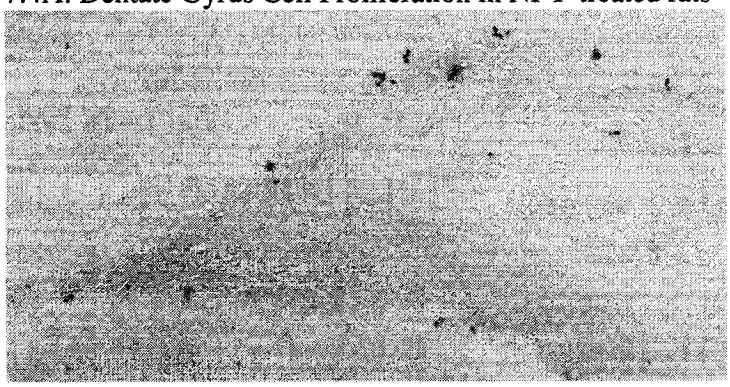


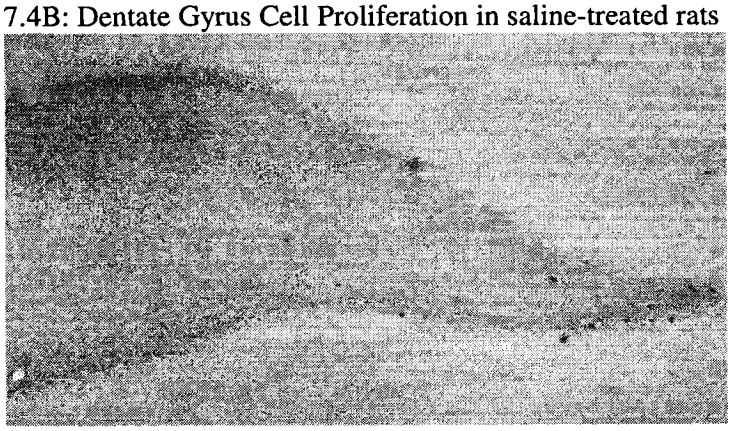
Figure 7.3: Proliferation in NPY, saline, and fluoxetine treated animals represented as the number of BrdU-positive cells in the dentate gyrus (7.3A) and SVZ (7.3B). Data are presented as mean ± SEM. Significant differences from saline. * $p < 0.05$ using between subjects ANOVA.

Figure 7.4

7.4A: Dentate Gyrus Cell Proliferation in NPY-treated rats



7.4B: Dentate Gyrus Cell Proliferation in saline-treated rats



7.4C: Dentate Gyrus Cell Proliferation in fluoxetine-treated rats

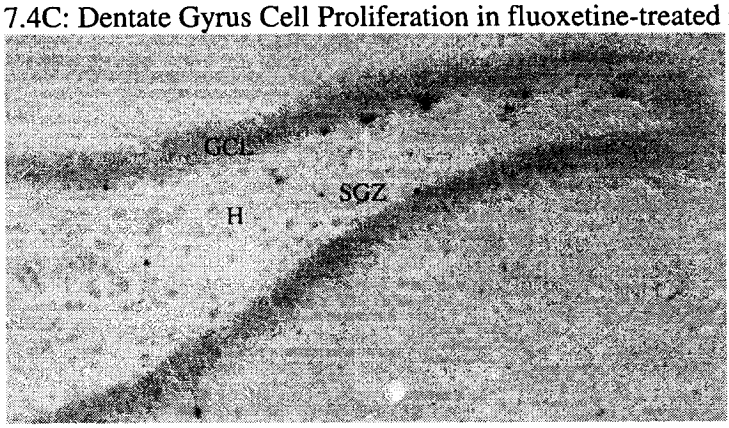
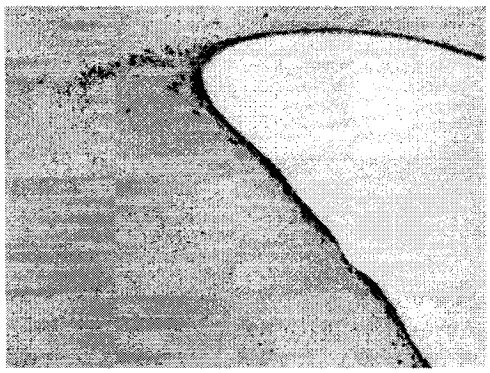


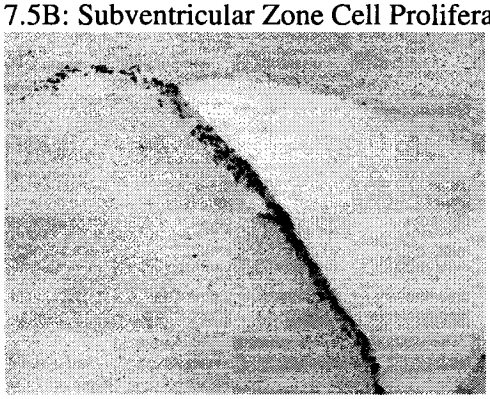
Figure 7.4: Representative photomicrographs (10x magnification) of BrdU-positive cells in the dentate gyrus of the hippocampus of NPY (7.4A), Saline (7.4B) and Fluoxetine (7.4C) treated rats visualized by DAB staining. The majority of the BrdU-labeled cells are located in the subgranular zone (SGZ), indicated by an *arrow* in 7.4C, the region between the granule cell layer (GCL) and hilus (H).

Figure 7.5

7.5A: Subventricular Zone Cell Proliferation in NPY-treated rats



7.5B: Subventricular Zone Cell Proliferation in saline-treated rats



7.5C: Subventricular Zone Cell Proliferation in fluoxetine-treated rats

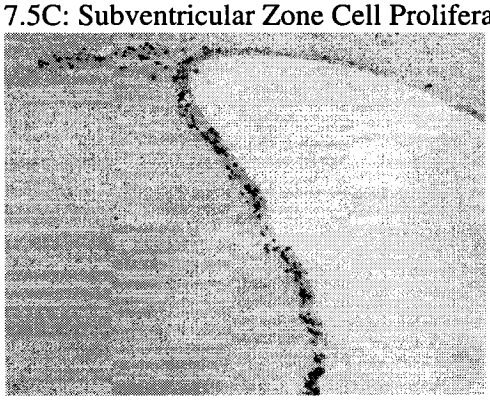


Figure 7.5: Representative photomicrographs (10x magnification) of BrdU-positive cells in the SVZ of NPY (7.5A), Saline (7.5B) and Fluoxetine (7.5C) treated rats visualized by DAB staining.

7.3 Discussion

This study demonstrates that chronic treatment with NPY exerts antidepressant-like effects in the rat forced swim test. Fourteen-day chronic treatment with NPY or fluoxetine in Sprague-Dawley rats significantly decreased immobility time in the forced swim test compared to saline-treated control animals. Treatment with NPY or fluoxetine did not significantly affect scores of passive swimming, active swimming/wall climbing or diving. The significant antidepressant-like effect in the forced swim test induced by NPY or fluoxetine do not appear to result from increased locomotor activity, as there was no significant difference in the number of total crossings in the open field test between groups.

The number of BrdU-positive neurons in the dentate gyrus was significantly higher in fluoxetine-treated animals compared to saline-treated control animals. This result is in agreement with previous studies that show fluoxetine has a significant effect on hippocampal proliferation [40]. The number of BrdU-positive neurons in NPY-treated animals was not significantly different from saline-treated control animals. However, the number of BrdU-positive neurons in fluoxetine-treated rats was not significantly different from the number of BrdU-positive neurons in NPY-treated rats. Although NPY did not increase hippocampal proliferation compared to saline-treated animals in this experiment, these results do suggest that NPY may have some role in hippocampal proliferation. Perhaps a higher dose of NPY is required to produce a significant increase in hippocampal proliferation. Another possibility is that NPY exerts antidepressant-like effects through a mechanism that is unrelated to hippocampal neurogenesis. For example, although the forced swim test has a significant degree of predictive validity, it may not

accurately screen for all possible antidepressant drugs with truly novel mechanisms of action.

There was no significant difference in BrdU-positive neurons in the SVZ between any of the groups. This result is also consistent with previous studies that show traditional antidepressant treatments like fluoxetine do not affect SVZ neurogenesis [40].

Although there was no significant change in hippocampal proliferation after chronic treatment with NPY compared to saline-treated animals, another factor of neurogenesis may have changed. Thus, the survival and differentiation of BrdU-positive neurons after chronic NPY treatment was examined next.

Chapter 8: Objective Three: Effect of chronic NPY treatment on emotional behavior and neurogenesis: Cell Survival and Differentiation

Regulation of neurogenesis can occur at several different stages including cell proliferation, differentiation and survival. Although there was no change in hippocampal proliferation after chronic treatment with NPY, there might be a difference in the differentiation or survival of newly born neurons. Consequently, the survival and differentiation of BrdU-labeled cells in the dentate gyrus was determined 3 weeks after BrdU administration and 14-days of chronic treatment with NPY, saline, or fluoxetine. The survival of SVZ BrdU-positive cells was also examined to determine if any effect of chronic NPY drug treatment on cell survival and differentiation are restricted to the hippocampus.

In this study, BrdU was given on days 1-4; osmotic pump implantation and drug treatment was started on day 7 and continued for two weeks. The last two days of treatment, rats were tested in the forced swim test and open field. The next day, the animals were sacrificed for a total survival time of 3 weeks.

8.1 Methodology

8.1.1 Animals

Sprague-Dawley rats n=9 [NPY], n=6 [saline], n=6 [Fluoxetine] were used in this experiment as described in *Section 7.1.1*

8.1.2 Drug Treatment

As described in *Section 7.1.2*

8.1.3 Forced Swim Test

As described in *Section 7.1.3*

8.1.4 Open Field Test

As described in *Section 7.1.4*

Neurogenesis: Cell Survival

8.1.5 Bromodeoxyuridine injections

In order to examine the survival of recently born neurons and determine the phenotypes of the individual neurons, rats received four daily injections of BrdU (75 mg/kg) prior to the 14-day drug treatment.

8.1.6 Perfusion and tissue storage

Rats were perfused 3 weeks after the 1st injection of BrdU as described in *Section 6.1.2*

8.1.7 Immunofluorescence labeling

To determine the phenotypes of BrdU-labeled neurons, every 6th section from each brain (n=7 [NPY]; n=6 [Saline]; n=6 [Fluoxetine]) was labeled for BrdU and NeuN (a marker for mature neurons). Sections were first pretreated by incubation in 2XSSC/50% formamide for 2 h at 65°C, rinsed in 2XSSC, incubated in 2M HCl for 30 min at 37°C and rinsed in borate buffer (0.1M, pH8.5) for 10 min. After blocking for 1 h in TBS (pH

7.5) containing 0.1% Triton X-100 and 3% normal donkey serum (TBS++), the sections were incubated overnight at 4°C in an antibody cocktail containing rat anti-BrdU monoclonal IgG (1:50; Accurate Chemical, Westbury, NY, USA) and mouse anti-NeuN monoclonal IgG (1:25; Chemicon, Temecula, CA, USA) in TBS++. The following day, the sections were rinsed in TBS, blocked for 10 min in TBS++ and incubated in a secondary antibody cocktail for 1 hour at room temperature in the dark. The cocktail contained Cy3-conjugated donkey anti-rat IgG (1:200; Jackson Immunoresearch, West Grove, PA, USA) and Cy2-conjugated donkey anti-mouse F(ab')2 fragment (1:50; Jackson Immunoresearch) in TBS++. The sections were rinsed in TBS, coverslipped with Aquapolymount (Polysciences, ces, Warrington, PA, USA) and stored at 4°C in the dark.

8.1.8 Quantification of BrdU labeling

Cells counts were conducted as described in *Section 6.1.4.* For phenotypic analysis, sections were viewed on a confocal laser scanning microscope (Nikon PCM 2000) with PCM 2000 software. Ten to twenty BrdU-labeled cells were identified per animal (n=7 [NPY]; n=6 [Saline]; n=6 [Fluoxetine]), and colocalization with NeuN was determined using Z-plane sectioning in single optical planes 1 μm thick.

8.1.9 Statistical Analysis

As described in *Section 7.1.6*

8.2 Results: Effect of Chronic NPY treatment on emotional behavior and neurogenesis: Cell Survival and Differentiation

Result 8.1: Forced swim test in NPY, saline, and fluoxetine-treated rats

Immobility

A between subjects analysis of variance (ANOVA) revealed a significant difference between groups in immobility during the forced swim test (Figure 8.1, Immobility; mean ± SEM: 16.25 ± 2.448 [NPY] vs. 35.67 ± 1.874 [Saline] vs. 21.00 ± 3.568 [Fluoxetine]; $F[2,17]= 13.91$, $p=0.0003$). A Tukey's multiple comparison post hoc test revealed significant differences between NPY and saline ($p < 0.001$) and Fluoxetine and saline ($p < 0.01$) but not between NPY and Fluoxetine ($p > 0.05$).

Passive Swimming

A between subjects analysis of variance (ANOVA) revealed a significant difference between groups in passive swimming during the forced swim test (Figure 8.1, Passive Swimming; mean ± SEM: 20.00 ± 3.202 [NPY] vs. 6.500 ± 1.384 [Saline] vs. 20.17 ± 4.102 [Fluoxetine]; $F[2,18]= 5.460$, $p= 0.0140$). A Tukey's multiple comparison post hoc test revealed significant differences between NPY and saline ($p < 0.05$) and Fluoxetine and saline ($p < 0.05$) but not between NPY and Fluoxetine ($p > 0.05$).

Active swimming/Wall climbing

A between subjects analysis of variance (ANOVA) did not reveal a significant difference between groups in active swimming or wall climbing during the forced swim test (Figure 8.1, Active Swimming/Wall climbing; mean ± SEM: 24.89 ± 2.874 [NPY] vs. 23.33 ± 2.704 [Saline] vs. 17.50 ± 2.592 [Fluoxetine]; $F[2,18]= 1.816$, $p=0.1912$).

Diving

A between subjects analysis of variance (ANOVA) did not reveal a significant difference between groups in diving during the forced swim test (Figure 8.1, Diving; mean ± SEM: 1.667 ± 0.4714 [NPY] vs. 0.8333 ± 0.6540 [Saline] vs. 0.0±0.0 [Fluoxetine]; $F[2,18]= 3.159$, $p=0.0667$).

Figure 8.1

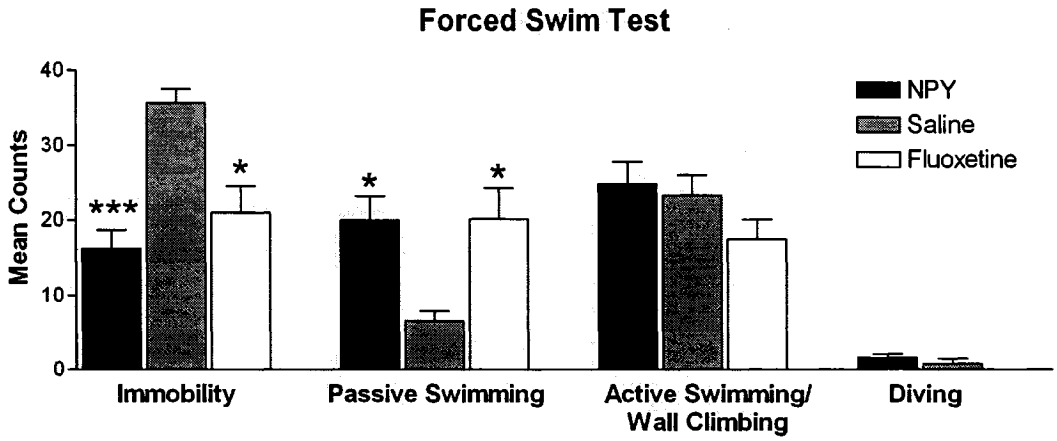


Figure 8.1: Behavior profile in the rat forced swim test after chronic treatment with NPY, saline, or fluoxetine. Behavior is recorded every 5 sec for 5 min. A lower immobility score is associated with positive antidepressant-like effects. Data are presented as mean ± SEM. Significant differences from saline: * $p < 0.05$, *** $p < 0.001$ using between subjects ANOVA

Result 8.2: Open field test in NPY, saline, and fluoxetine-treated rats

A between subjects analysis of variance (ANOVA) did reveal a significant difference between groups in total crossings during the open field test (Figure 8.2, Total Crossings; mean ± SEM: 174.8 ± 19.29 [NPY] vs. 240.0 ± 33.59 [Saline] vs. 137.5 ± 19.17 [Fluoxetine]; $F[2,18]= 4.084$, $p=0.0345$). A Tukey's multiple comparison post hoc test revealed significant differences between saline and fluoxetine ($p < 0.05$) but not NPY and Saline ($p > 0.05$) or NPY and Fluoxetine ($p > 0.05$).

Figure 8.2

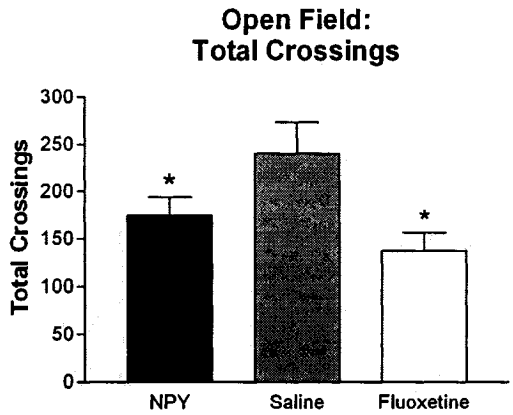


Figure 8.2: Locomotor activity in the open field test after chronic treatment with NPY, saline, or fluoxetine. Total crossings were recorded for 10 min. Data are presented as mean ± SEM. Data analyzed using between subjects ANOVA * $p < 0.05$ using between subjects ANOVA

Result 8.3A: Dentate Gyrus cell survival in NPY, saline, and fluoxetine-treated rats

A between subjects analysis of variance (ANOVA) did not reveal a significant difference between number of BrdU-positive cells in the dentate gyrus (Figure 8.3A, Dentate Gyrus Cell Survival; mean ± SEM: 1263 ± 199.0 [NPY] vs. 950.4 ± 199.5 [Saline] vs. 1347 ± 316.1 [Fluoxetine]; $F[2,16]= 0.5878$, $p= 0.5671$).

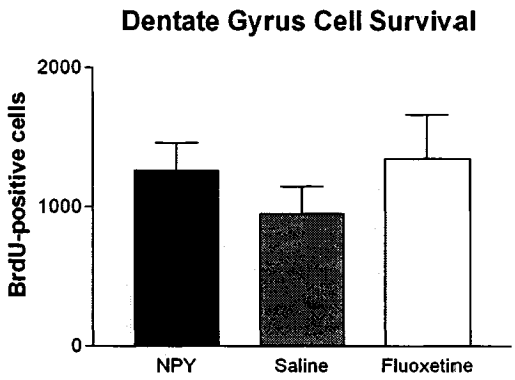
Result 8.3B: Subventricular Zone cell survival in NPY, saline, and fluoxetine-treated rats

A between subjects analysis of variance (ANOVA) did not reveal a significant difference in the number of BrdU-positive cells in the subventricular zone between groups (Figure 8.3B, SVZ Cell Survival; mean ± SEM: 3185 ± 452.8 [NPY] vs. 1883 ± 338.3 [Saline] vs. 1773 ± 458.2 [Fluoxetine]; $F[2,16]= 3.362$, $p=0.0604$).

The survival of newly born cells in the dentate gyrus and subventricular zone was determined using double labeling immunofluorescence and visualized with Cy3 fluorescence (Figure 8.4 and Figure 8.5). BrdU-positive cells (red) are present in the dentate gyrus three weeks after BrdU administration and 14 day chronic treatment with NPY (8.4A), saline (8.4B) or fluoxetine (8.4C). BrdU-positive cells (red) are also present in the subventricular zone three weeks after BrdU administration and 14 day chronic treatment with NPY (8.5A), saline (8.5B) or fluoxetine (8.5C).

Figure 8.3

8.3A



8.3B

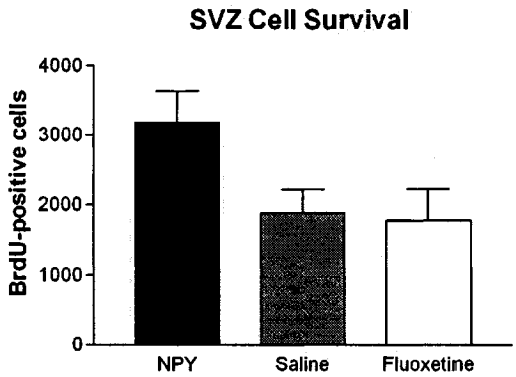
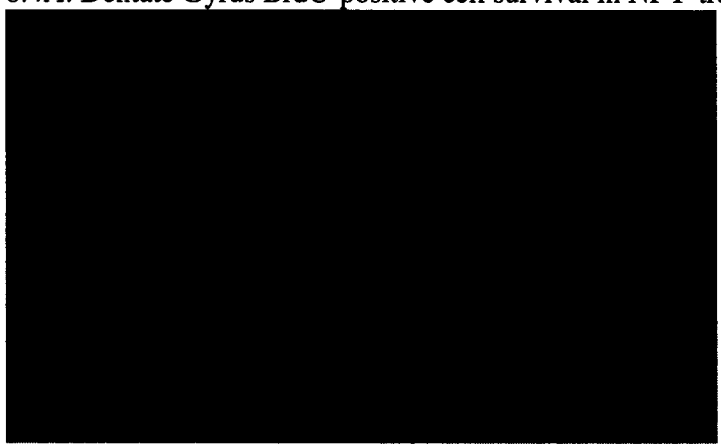


Figure 8.3: Survival in NPY, saline, and fluoxetine-treated animals represented as the number of BrdU-positive cells in the dentate gyrus (8.3A) and SVZ (8.3B). Data are presented as mean ± SEM. Data analyzed using between subjects ANOVA.

Figure 8.4

8.4A: Dentate Gyrus BrdU-positive cell survival in NPY-treated rats



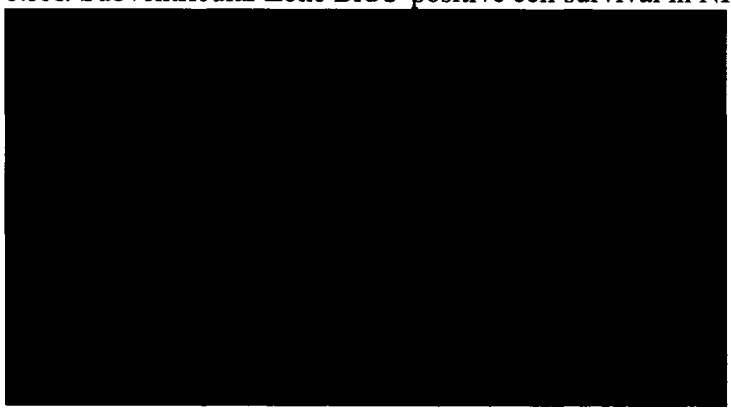
8.4B: Dentate Gyrus BrdU-positive cell survival in saline-treated rats

8.4C: Dentate Gyrus BrdU-positive cell survival in fluoxetine-treated rats

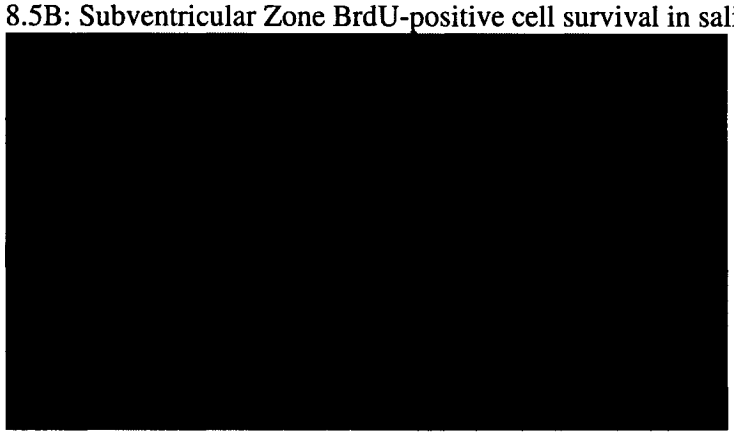
Figure 8.4: BrdU-positive cells (red) in the dentate gyrus of the hippocampus (10x magnification) of NPY (8.4A), saline (8.4B) and fluoxetine (8.4C) treated rats visualized with Cy3 fluorescence.

Figure 8.5

8.5A: Subventricular Zone BrdU-positive cell survival in NPY-treated rats



8.5B: Subventricular Zone BrdU-positive cell survival in saline-treated rats



8.5C: Subventricular Zone BrdU-positive cell survival in fluoxetine-treated rats

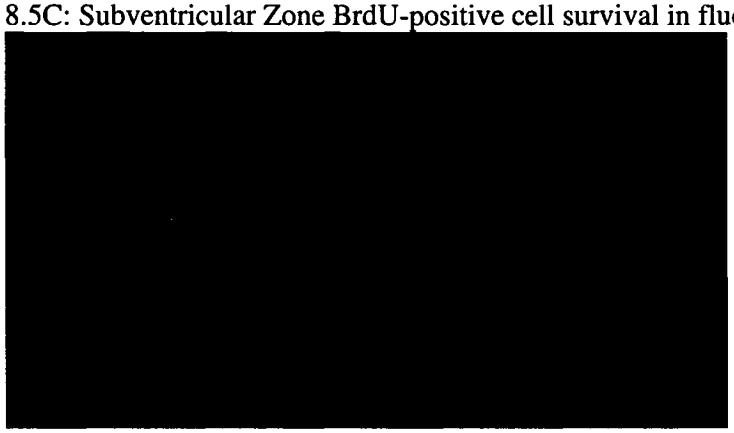


Figure 8.5: BrdU-positive cells (red) in the SVZ (10x magnification) of NPY (8.5A), saline (8.5B) and fluoxetine (8.5C) treated rats visualized with Cy3 fluorescence.

Result 8.6: Phenotype of BrdU-positive cells in NPY, saline, and fluoxetine-treated rats

A between subjects analysis of variance (ANOVA) did not reveal a significant difference in the percentage of $BrdU^+/NeuN^+$ cells in the dentate gyrus between groups (Figure 8.6, Percentage of $BrdU^+/NeuN^+$ cells; mean ± SEM: 77.85% ± 3.836 [NPY], 84.39% ± 5.927 [saline], and 89.77% ± 2.071 [fluoxetine]; $F[2,16]= 2.059$, $p=0.16$).

The phenotype of newly born cells in the dentate gyrus was determined using double labeling immunofluorescence and visualized with a confocal microscope (Figure 8.7). BrdU-positive cells (red) and NeuN-positive (green) cells are present in the granule cell layer of the dentate gyrus three weeks after BrdU administration and 14 day chronic treatment with NPY (8.7A), saline (8.7B) or fluoxetine (8.7C). Colocalization of BrdU and NeuN indicate that the newly born cells express a neuronal phenotype.

Figure 8.6

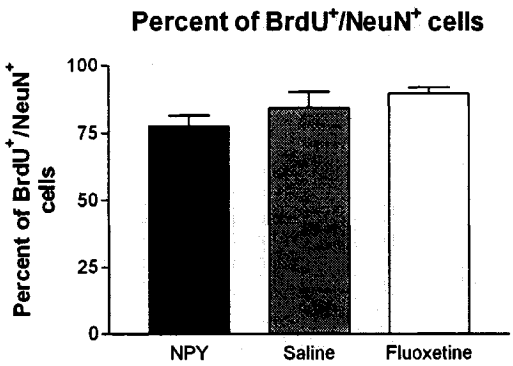
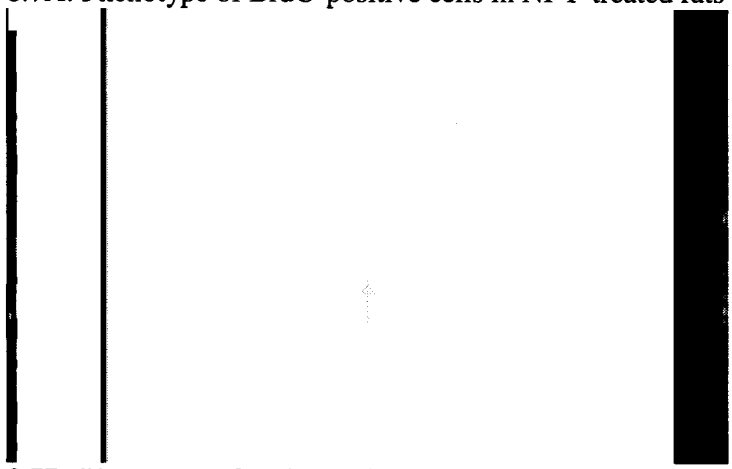


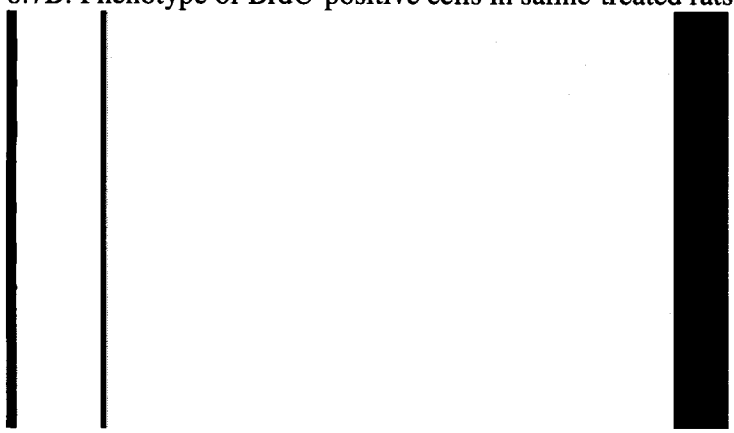
Figure 8.6: Percentage of BrdU-positive/NeuN positive cells in the dentate gyrus of NPY, saline, and fluoxetine-treated rats. Data are presented as mean ± SEM. Data analyzed using between subjects ANOVA.

Figure 8.7

8.7A: Phenotype of BrdU-positive cells in NPY-treated rats



8.7B: Phenotype of BrdU-positive cells in saline-treated rats



8.7C Phenotype of BrdU-positive cells in fluoxetine-treated rats

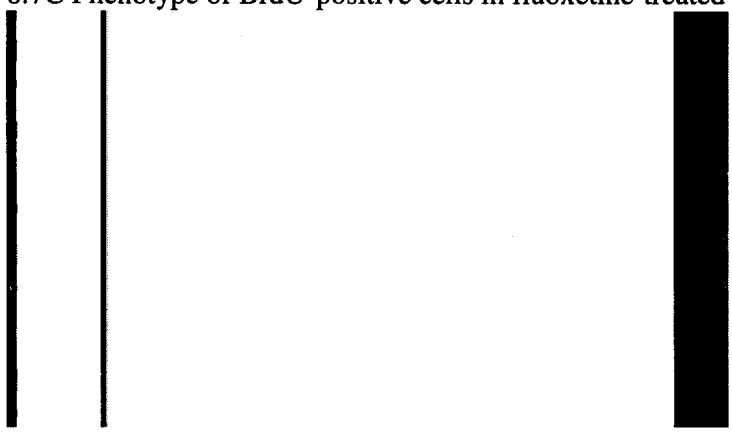


Figure 8.7: Representative confocal laser-scanning image (60x magnification) of BrdU-positive (red) and NeuN-positive (green) cells in the GCL of the dentate gyrus three weeks after BrdU administration and 14 day chronic treatment with NPY (8.7A), saline (8.7B) or fluoxetine (8.7C). Colocalization is represented by the *arrow* in 8.7A.

8.3 Discussion

The regulation of neurogenesis at the level of differentiation and survival of BrdU-positive cells was examined in this study. Fourteen-day chronic treatment with NPY and fluoxetine had a significant decrease on immobility time and passive swimming in the forced swim test compared to saline-treated animals. The significant antidepressant-like effect in the forced swim test does not appear to result from increased locomotor activity because the number of total crossings in the open field test by NPY and fluoxetine-treated animals was actually decreased compared to saline-treated animals. Although the reason for this is unknown; it does not affect the interpretation of the antidepressant-like profile of NPY- and fluoxetine-treated animals.

Three weeks after treatment with BrdU and two weeks of chronic treatment with NPY, saline, fluoxetine, there was no difference in the survival of BrdU-positive cells in the dentate gyrus of the hippocampus. Interestingly, there was a trend towards an increase in the survival of BrdU-positive neurons in the SVZ of the NPY group compared to both saline and fluoxetine-treated animals. This is interesting as it has been shown that seizure can increase SVZ neurogenesis [260] and there is extensive evidence for NPY and seizure [261].

The phenotype of BrdU-labeled cells was determined with the neuronal phenotypic marker NeuN in the dentate gyrus. In the SGZ, progeny migrate outward to the granular cell layer and differentiate into neurons [72]. The majority of the BrdU positive neurons born in the SVZ migrate anteriorly through the rostral migratory stream into the olfactory bulb where they mature into interneurons [71]. Therefore, the differentiation of BrdU-positive cells was determined in the dentate gyrus only. In this study, the majority (77% ± 4 [NPY], 84% ± 6 [saline], and 90% ± 2 [fluoxetine]) of surviving BrdU-positive cells

expressed a neuronal marker NeuN. Colocalization of BrdU and NeuN confirms that BrdU-positive cells mature into neurons. The other percentage of BrdU-positive cells are either glial or may represent another phenotype. This finding (~83.68 ± 2.573 $BrdU^+/NeuN^+$ cells) is in agreement with other studies that found ~75% $BrdU^+/NeuN^+$ cells [70;76]. The results of the differentiation study indicate that the percentage of $BrdU^+/NeuN^+$ cells are not influenced by chronic treatment with NPY, saline, or fluoxetine. This result is also in agreement with a study that found no change in the differentiation of BrdU-positive neurons after chronic treatment with fluoxetine [40].

Chapter 9: General Discussion and Conclusion

It is possible that abnormalities in neuroplasticity are the direct result of lifetime experiences (such as chronic stress) with functional consequences that ultimately disrupt normal cellular processes and lead to mood disorders such as depression. In fact, depression is traditionally viewed as the manifestation of an inability to cope with various lifetime stressors [55;262] that is presumably determined by genetics. Given the significance of NPY in the central nervous system as a modulator of emotional behavior and the recent discovery of its role in neuroplasticity; further investigation of NPY demonstrated a sensible approach to elucidate the mechanisms involved in the etiology of depression and/or the effective reversal of symptoms.

The results of these studies confirm that NPY has a significant role in emotional behavior. For example, young and old NPY transgenic rats displayed anxiolytic-like behavior in the open field and were resistant to acute physical restraint stress measured by the elevated-plus maze (Please see *Appendix A).* In addition, NPY Y_2 knockout mice had a significant anxiolytic-like phenotype in the elevated-plus maze and open field test compared to control mice. Although no difference in emotional behavior was observed in NPY Y_1 knockout and control animals, there is still compelling evidence for NPY as an endogenous anxiolytic. The NPY system offers an attractive target for drug development. Orally available non-peptide, small molecule antagonists of the Y_2 receptor that are effectively able to penetrate the CNS are pharmaceutical targets that might be very successful in the treatment of anxiety and anxiety-related disorders in human subjects.

The results of these studies also reveal that NPY Y_2 knockout mice had lower immobility scores in the forced swim test compared to control mice; indicating potential antidepressant-like properties of NPY. Interestingly, depression with psychiatric co-

morbidity, particularly anxiety, has been found to be associated with greater severity of depression and anxiety, poorer or delayed response to antidepressants, functional impairment and decreased responsiveness to treatment [reviewed in [263]]. Consequently, NPY presents a novel approach in the search for effective antidepressant treatment strategies given its significant role in both anxiety and depression-related behaviors. Although full characterization of the role of NPY in the pathophysiology of depression is still required, additional studies using transgenic and knockout models as well as the development of double and triple receptor knockout mice [214] will help determine the validity of NPY as a therapeutic target molecule for the treatment of mood disorders such as depression.

There is increasing evidence that abnormalities in hippocampal neurogenesis are involved in the pathophysiology and/or treatment of mood disorders such as depression. Therefore, several objectives in this study were aimed at characterizing the endogenous function of NPY as a modulator of neuroplasticity; specifically neurogenesis, within the SVZ and hippocampus. Hippocampal proliferation was first examined in NPY Y_2 receptor subtype knockout mice and wildtype controls. Although no significant difference between the number of BrdU-positive cells in the dentate gyrus or SVZ of NPY Y_2 KO mice and wildtype controls was found, the age of the mice may have negatively influenced the level of neurogenesis in these mice so that a significant difference between groups could not be observed. Hence, future studies should examine cell proliferation and survival in young (~3 month) Y_2 KO and wildtype controls. Cell proliferation and survival should also be examined in Y_5 KO mice in order to determine the role of this receptor subtype in hippocampal and SVZ neurogenesis.

In the third and forth objectives, the effect of chronic NPY treatment on emotional behavior and neurogenesis (proliferation, survival, and differentiation) in the rat was determined. In both studies, 14-day chronic treatment with NPY and fluoxetine significantly decreased immobility time in the forced swim test compared to saline-treated control animals. The significant antidepressant-like effect in the forced swim test does not appear to result from increased locomotor activity based on data from the open field. There was no significant difference in the number of total crossings between groups in the proliferation study while in the survival study; the number of total crossings by NPY and fluoxetine-treated animals was decreased compared to saline-treated animals.

The number of BrdU-positive neurons in the DG was significantly higher in fluoxetine-treated animals compared to saline-treated control animals. There was no significant difference in SVZ neurogenesis between groups. This result is in agreement with previous studies that show fluoxetine has a significant effect on hippocampal but not SVZ proliferation [40]. The number of BrdU-positive neurons in NPY-treated animals was not significantly different from saline-treated control animals. However, the number of BrdU-positive neurons in fluoxetine-treated rats was not significantly different from the number of BrdU-positive neurons in NPY-treated rats. Although NPY did not increase hippocampal proliferation compared to saline-treated animals in this experiment, these results do suggest that NPY may have some role in hippocampal proliferation. A higher dose of NPY may be required to significantly increase hippocampal proliferation. However, NPY-related drugs may still be beneficial (regardless of their effect on neurogenesis) because of their significant anxiolytic and antidepressant-like effects.

There was no difference in the survival of BrdU-positive cells in the dentate gyrus of the hippocampus after 14-days chronic treatment with NPY, saline, or fluoxetine. Interestingly, there was a trend towards an increase in survival of BrdU-positive neurons in the SVZ of NPY-treated animals compared to saline and fluoxetine-treated animals. These results are consistent with previous studies that show traditional antidepressants like fluoxetine do not affect dentate gyrus or SVZ survival [40]. The role of NPY in SVZ neurogenesis should be explored in future experiments. The results of the differentiation study indicate that the percentage of BrdU/NeuN-positive cells is not significantly different between groups after chronic treatment with NPY, saline or fluoxetine. This result is also in agreement with a study that found no change in the differentiation of BrdU-positive neurons after chronic treatment with fluoxetine [40].

Overall, the results of these studies do not fully support a role for NPY in the modulation of neurogenesis in the dentate gyrus or SVZ. However, the role for NPY in neurogenesis in relation to mood disorders cannot be completely ruled out. For example, tissue penetration may be poor with NPY peptide. Therefore, chronic treatment with Y_1 agonists or Y_2 antagonists might have a significant effect on behavior as well as hippocampal proliferation. Future studies should examine the effect of pharmacological drugs aimed at specific receptor subtypes on neurogenesis in the hippocampus as well as other regions such as the olfactory bulb. Another possibility that should be explored is the ability of NPY and its receptor subtypes to stimulate hippocampal proliferation in animal models of depression such as in OBX or chronic mild stress. A "depressed" phenotype may be required for NPY to significantly increase neurogenesis in the hippocampus.

Conversely, there have been several studies that have shown proliferation and corresponding emotional behavior are not necessarily related. For example, decreasing hippocampal proliferation with restraint stress or an active avoidance task was not correlated with the development of learned helplessness or "depressed" behavior [264]. In this study, there was no difference in levels of hippocampal proliferation between rats who were learned helpless and those who were resistant; suggesting that decreasing neurogenesis does not inevitably lead to "depressed" behavior [264]. Another study has shown that partial serotonin depletion in the adult rat brain with a single injection of *para*-chloroamphetamine (PCA) reduces hippocampal proliferation but has no change on immobility time in the forced swim test [265]. Moreover, reducing hippocampal neurogenesis by 90% relative to sham controls with irradiation had no significant difference in the baseline behavior in the novelty suppressed feeding test [62]. These studies weaken the theory that impaired hippocampal neurogenesis has a causal role in depression and that antidepressant drugs normalize the deficit in hippocampal neurogenesis. These studies also express some degree of doubt as to the genuine role of hippocampal neurogenesis in depression. Thus, NPY may actually have antidepressant-like activity in the forced swim test that is unrelated to hippocampal proliferation.

In conclusion, NPY has a significant role in the modulation of emotional behavior in animal models. These studies could not confirm that chronic treatment with NPY exerts its antidepressant-like effects in the forced swim test through hippocampal proliferation. Additional research is required to determine the role of NPY in DG and SVZ neurogenesis and to elucidate the mechanism by which NPY exerts its antidepressant-like activity in the forced swim test.

Chapter 10: References

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Chapter 11: Appendices

Appendix A: Aged neuropeptide Y transgenic rats are resistant to acute stress but maintain spatial and non-spatial learning and memory

Reprinted from Behavioral Brain Research, 153, Carvajal, C.C., Vercauteren, F., Dumont, Y., Michalkiewicz, M., & Quirion, R, Aged neuropeptide Y transgenic rats are resistant to acute stress but maintain spatial and non-spatial learning, 471-480, Copyright 2004, with permission from Elsevier.

Contributions of Authors:

Cristina C Carvajal completed the behavioral analysis (including the elevated plus maze, open field test, Morris water maze, and object recognition test), analyzed the data, and prepared the manuscript.

Freya Vercauteren completed the delayed alternation version of the Morris water maze.

Yvan Dumont provided guidance in the preparation and revision of the manuscript.

Michael Michalkiewicz developed the NPY transgenic rat and supplied our laboratory with male NPY transgenic and wildtype control rats.

Rémi Quirion assisted in the preparation and revision of the manuscript. Dr. Rémi Quirion also provided financial support.

Aged neuropeptide Y transgenic rats are resistant to acute stress but maintain spatial and non-spatial learning and memory

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Abstract

The behavioral phenotype of five-month-old rats overexpressing neuropeptide Y (NPY) has previously been described [Proc Natl Acad Sci USA 97 (2000) 12852]. In this transgenic rat model, there is central overexpression of prepro-NPY mRNA and NPY peptide in the hippocampus and hypothalamus and decreased Y_1 binding sites within the hippocampus. These molecular and neurochemical events led to altered anxiety profile and learning abilities in NPY-overexpressing rats. In the present study, anxiety and learning/memory related behaviors were examined in one-year-old NPY-transgenic rats in order to assess any behavioral changes that may have occurred during the aging process. As observed in 5-month-old overexpressing rats, aged NPY-transgenic animals are resistant to acute physical restraint stress measured by the elevated-plus maze and demonstrate anxiolytic-like activity in the open field. However, in contrast to data in young rats, there was no significant difference between aged wildtype and NPY-transgenic animals in relation to spatial and non-spatial memory as indicated by the (allo- and ego-centric) Morris water maze and object recognition test. It would thus appear that the anxiolytic-like profile observed in young NPY-overexpressing rats is maintained in older animals providing further evidence for a role for NPY in anxious behaviors. However, the cognitive deficits observed in young rats do not appear to occur in older animals suggesting the existence of compensatory mechanisms leading to a reversal of the learning deficits noted in younger animals. These results also provide additional evidence for the mechanistic dissociation between anxiety and cognition-related behaviors modulated by NPY.

Keywords: Neuropeptide Y; Learning; Anxiety; Water maze; Object recognition; Transgenic rat

1. Introduction

Neuropeptide Y (NPY) is a 36 amino acid peptide that is widely distributed in the central nervous system [16]. It is one of the most conserved peptides in evolution, suggesting an important role in the regulation of basic physiological functions [36]. The various biological effects of NPY can be mediated by the activation of the Y_1, Y_2, Y_4, Y_5, and y_6 receptor subtypes [46]. Interestingly, however, the y6 receptor is not expressed in the rat [53] while in humans and primates; the y_6 cDNA contains a single base pair deletion resulting in the expression of a non functional receptor protein [40]. NPY and its receptors are widely distributed in the brain and have been implicated in various biological processes [16]. For example, NPY is one of the most potent peptides to induce feeding by activating Y_1 and/or Y_5 subtypes [3,33]. Studies in transgenic animals have revealed that NPY is implicated in alcohol intake (mediated by the Y_1 subtype) [70] and seizure activity (mediated by Y_1, Y_2, and Y_5 subtypes) [66,76]. Furthermore, NPY and Y_1 receptors have been associated with depression [60] and anxiety related behaviors [28]. Moreover, there is evidence that NPY and Y_2 receptor subtypes have a role in modulation of learning and memory processing [50,58,61]. NPY has also been implicated as a neuroproliferative factor in post natal hippocampal precursor cells [32].

NPY is believed to influence the manner in which a subject responds to stress. Preclinical and clinical evidence suggests that NPY is involved in the regulation of anxiety related behaviors [22,27,28,59]. NPY is consistently reported to elicit anxiolytic-like effects in models of anxiety including punished responding tests [6], exploratory behavior-based tests [1] social interaction [62], and fear-potentiated startle [8]. In clinical studies, there is a positive association between acute, uncontrollable stress and robust

increases in plasma levels of NPY [48,49]. In addition, a correlation between higher levels of stress-related NPY release and lower levels of subjective psychological distress has been reported [47,48]. In contrast to the extensive and consistent evidence for NPY in the regulation of anxiety, the role of NPY in learning and memory-related behaviors is unclear.

Initial studies demonstrated that NPY displayed antiamnesic effects in mice treated with the protein synthesis inhibitor, anisomycin, or the muscarinic receptor antagonist, scopolamine [20]. More recently, NPY was shown to attenuate learning impairments induced by the non-competitive NMDA receptor channel antagonist, dizocilpine (MK-801) [5]. Evidence for a physiological role of NPY in cognitive behaviors was first demonstrated in passive immunization studies with NPY antibodies injected into the hippocampal region and the induction of amnesia [19]. Additional experiments have revealed that the effects of NPY on cognitive function are region specific. Injection of NPY into the rostral hippocampus and the septal area was shown to enhance memory retention, whereas NPY injection into the amygdaloid body and the caudal hippocampus induced amnesia [19]. Most recently, Redrobe et al. [58] reported cognitive deficits in NPY Y_2 receptor knockout mice.

The development of an NPY-transgenic rat has provided an opportunity to study the effects of this peptide on learning and memory processing. Anatomical mapping studies of these animals have revealed highly significant overexpression of hippocampal NPY in the CA1 region of the young NPY-transgenic animals [73]. Recently, NPY protein levels were also shown to be significantly higher in the paraventricular, suprachiasmatic and supraoptic nuclei of the hypothalamus and tended to be increased in the arcuate nucleus

in these rats [43,45]. The transgenic animals were generated using a 14.5-kb fragment of the rat NPY genomic sequence including normal intronic sequence elements. It is flanked by an approximate 5-kb 5' sequence thought to contain the major regulatory elements normally controlling NPY expression [44]. Consequently, the regulation of the NPY transgene is predicted to be similar to the wildtype of endogenous NPY.

The behavioral phenotype of transgenic rats overexpressing NPY has previously been characterized as insensitive to restraint stress with an absence of fear suppression and impaired spatial learning [73]. However, in aged animals, a reduced level of NPY peptide concentration in the dentate gyrus of the hippocampus compared to young animals has been reported [30,74]. Hilar NPY-immunoreactive interneurons in the dentate gyrus were also shown to be significantly decreased in aged rats compared to young controls [9]. Although deficits in spatial cognition in aged animals is common [57], no correlation has been found between the degree of hilar NPY interneuron loss and spatial performance in the Morris water maze [9]. In addition, decreases in hilar NPY-interneurons have been reported in tissue from patients with Alzheimer's Disease [10].Consequently, the effect of NPY overexpression on behaviors such as cognition in aged animals is unknown. In order to assess the role of aging on the behavioral phenotype of NPY-transgenic rats, the present study investigated anxiety and cognitive behaviors in year-old animals. Given that the hippocampus is a key structure in learning and memory processing and in the modulation of anxiety [38,42] anxiety was measured in the elevated plus maze and the open field and potential spatial and non-spatial learning/memory deficits were examined in the Morris water maze, the delayed alternation version of the Morris water maze, and the object recognition test.

2. Materials and methods

2.1. Animals

The generation of NPY-transgenic Sprague–Dawley rats has been described by [44,73] using a 14.5-kpb lambda construct containing the entire rat NPY gene. Animals obtained from the Medical College of Wisconsin at 6 months of age and were housed in pairs in the Douglas Hospital Research Center facilities under standard laboratory conditions (12 h light/12 h dark cycle lights on at 07:00 h, food and water ad libitum). Animal care was provided according to protocols and guidelines approved by McGill University and the Canadian Council of Animal Care. These animals had no prior behavioral or pharmacological testing. The elevated plus maze testing was performed first, followed by the open field test two days later. The Morris water maze was performed one week later. The object recognition task was completed two months after the Morris water maze and the delayed alternation task was carried out two months later. All transgenic and wildtype animals were subjected to each of the described behavioral tests, with the exception of one transgenic animal during the delayed alternation task.

2.2. Morris water maze task

The test was performed as previously described [51,58]. The experimental apparatus consisted of a circular pool (diameter: 120 cm) filled with tap water, made opaque with powdered milk, and was maintained at 24 ±1 °C. The escape platform (diameter: 8 cm) was hidden 0.5 cm below the surface of the water and remained in a fixed position throughout the acquisition training. NPY-transgenic (n = 10) and wildtype rats (n = 12) were given four trials per day over four consecutive days (16 trials in all). For each trial, the rat was placed in the pool (facing pool wall) at one of four selected starting points

(north, south, east, or west quadrant). On locating the platform, the rat was allowed to remain there for 15 s before being returned to its home cage. If the rat did not find the platform within 120 s, it was set on the platform by hand and allowed to remain there for 15 s and the escape latency for that trial was recorded as 120 s. The total escape latency (average for all trials per day) and swim speed were measured by a video tracking system connected to a computer equipped with the commercially available HVS image system (HVS, UK) for the analysis of Morris water maze performance. On the fifth day, a probe trial (120 s) was performed with the platform removed from the pool. The rats were then subjected to another trial in which the platform (placed in its original position) was made visible above the water surface.

2.3. Delayed alternation version of the Morris water maze

The test was performed as previously described [2,18,37]. In this variant of the Morris water maze, the hidden target platform alternated between two locations (north-east and north-west quadrant of the pool), while the animals always entered the pool in the southern quadrant. Four months after the original Morris water maze task and two months after the object recognition task, the same NPY-transgenic (n = 9) and wildtype rats (n = 12) were submitted to six trials per day for five consecutive days with a cut-off time of 60s and an average intertrial interval of 15 min. On locating the platform, the rat was allowed to remain there for 15 s before being returned to its home cage. The escape latency, quadrant latencies and swim speed were measured by a video tracking system connected to a computer equipped with the commercially available HVS image system (HVS, UK). The escape latencies per trial during the acquisition phase were used for statistical analysis to test memory acquisition and to test whether there was an effect per

trial. The total escape latencies for all trials per day were used for statistical analysis to measure the effect per day. On the sixth day, a probe trial (60 s) was performed, as well as a visual cued test in which the platform (placed in the south–west quadrant) was made visible above the water surface, while rats entered the pool in the eastern quadrant.

2.4. Object recognition test

The test employed was essentially similar to that described elsewhere [17,58]. NPY-transgenic (n = 10) and wildtype (n = 12) rats were tested for object recognition in clear plastic animal cages. For each animal, one pair of objects was selected at a random from a set of four objects that differed in shape, surface color, contrast, and texture. The four objects were selected from a larger pool of objects based on the criterion that rats would spend approximately equal time exploring each of the objects. Rats were habituated to the test environment over three daily sessions of 15 min. On the test day, two identical objects were placed on the centerline of the long axis of the chamber floor, 5 cm from each cage end. Rats were allowed to explore the two objects for 5 min and exploratory activity (i.e., the time spent exploring each object was recorded). After a delay of 6 h, rats were re-exposed to a familiar object (from acquisition phase), together with a novel object (not used in acquisition phase). Once again, the time that each animal spent exploring each object was measured. A rat was considered to be engaging in exploratory behavior if the animal touched the object with its forepaw or nose, or sniffed at the object within a distance of 1.5 cm. The choice of object for novel or familiar was counterbalanced and the position of each object was also alternated between trials to avoid any misinterpretation of data. After each exposure, the objects and test chamber were cleaned with 70% ethanol to eliminate odor cues. A memory index (MI) was

calculated for each rat, where "*t*o" represented time exploring the familiar object (from acquisition phase) and "*t*n" the time exploring the novel object (from recognition phase): MI = *(t*n – *t*o*)/(t*n + *t*o*)* [14].

2.5. Elevated plus-maze

The test was performed as previously described [24,25,54,59]. The experimental apparatus consisted of a plus-formed maze elevated 50 cm above the ground. The four arms were 50 cm long and 10 cm wide. Two opposing arms were surrounded by black Plexiglas walls 15 cm high (closed walls); while the other arms were devoid of walls (open arms). Each rat (n = 10 transgenic; n = 12 wildtype) was placed in the center of the maze facing an open arm, after which the cumulative time spent in each arm and the number of entries into the open or closed arms were recorded during a 5 min test session. An individual entry into the arm was defined as the animal placing all four paws in that arm. The time spent in the open arms is expressed as a percentage of the total time spent in the arms (% time), and the number of entries in the open arms as a percentage of the total numbers of entries (% entry). The data is shown graphically as a percentage of the (open/(open+closed)) in both the number of entries and time spent in the open arms (% open) as well as number of entries into the open arms and time spent on the open arms. Total number of entries onto any arm is presented as a measure of general locomotor activity on the maze so as to rule out any non-specific effects that may have interfered with the interpretation of the data. Each animal was tested in the plus maze for baseline measures and one week later the animals were presented with a one-hour physical restraint stress challenge and one hour later re-tested in the maze.

2.6. Open field test

In order to test locomotor activity, NPY-transgenic (n = 10) and wildtype (n = 12) rats were tested in the open field [23,59]. Rats were placed into the center of the apparatus, which consisted of a square base (70 cm×70 cm) surrounded by a 75 cm high wall. Illumination was provided by a 40W bulb, positioned 90-cm above the floor of the apparatus. Animals were placed into the center of the apparatus and the time spent in the central area of the arena is expressed as a percentage of the total time (% time). The number of crossings into the central area is expressed as a percentage of the total number of crossings (% entry). Total crossings are presented as a measure of general locomotor activity in the arena, so as to rule out any non-specific effects that may have interfered with the interpretation of data. Testing was conducted over a 10-min period and was recorded by a video tracking system connected to a computer equipped with the commercially available HVS image system (HVS, UK) for the analysis of the open field activity.

2.7. Statistics

Results are expressed as means ±SEM for Morris water maze escape latency, alternation escape latency, object recognition MI, plus-maze % entries, % time on both open and closed arms, and % entries, % time in central area and total crossings of the open field. Student's t-tests were used to assess statistical differences between groups. Results in the elevated plus maze as well as differences in escape latency in the Morris water maze and delayed alternation test were calculated using analysis of variance (ANOVA) with repeated measures (Excel, SPSS; $P < 0.05$ considered statistically significant; n = 10 for aged NPY-transgenic rats and n = 12 for aged matched wildtype rats. For delayed

alternation test, n = 9 for aged NPY-transgenic rats and n = 12 for age matched wildtype rats).

3. Results

3.1. Elevated plus maze

The percentage of open arm entries and the percentage of time spend in the open arms is expressed graphically as % open (open/(open + closed)) (Fig. 1). Baseline values for the percentage of open entries (t (20) = 0.45; p = 0.66; unpaired t-test; mean ± SEM: 0.34 ± 0.09 wildtype versus 0.40 ± 0.10 NPY-transgenic) and percentage of time spend in the open arms were not significant (t (20) = 0.204; p = 0.84; unpaired t-test) between the NPY-transgenic and wildtype groups (mean ± SEM: 0.23 ± 0.06 wildtype versus 0.24 ± 0.038 NPY-transgenic) in the elevated plus maze. However, a pretest exposure in the form of one-hour physical restraint stress revealed significant differences in the open arm activity. A highly significant anxiogenic effect of the restraint was seen in the wildtype controls. The percentage of entries onto the open arms following restraint stress (mean ± SEM: 0.34 ± 0.09 no restraint versus 0.09 ± 0.038 restraint) is significant for wildtype rats (t (11) = 3.92; p = 0.0024; paired t-test), but not significant for NPY-transgenic rats (t (9) = 0.33; p = 0.75; paired t-test; mean ± SEM: 0.41 ± 0.10 no restraint versus 0.36 ± 0.06 restraint) when compared to the percentage of entries prior to restraint stress. The percentage of time spent on the open arms following restraint stress (mean ± SEM: 0.23 ± 0.06 no restraint versus 0.7 ± 0.03 restraint) is significant for wildtype rats (t (11) = 2.33; p = 0.039) but the percentage is not significant for NPY-transgenic animals (t (9) = 2.59; p = 0.80) when compared to non-stress conditions (mean ± SEM: 0.25 ± 0.04 no restraint

versus 0.23 ± 0.02 restraint; n = 10 for aged NPY-transgenic rats and n = 12 for aged matched wildtype rats).

The number of open and closed arm entries and time spent in each arm is represented in Table 1. Baseline values for the number of open entries (t (20) = 1.36; p = 0.188) (mean ±SEM, 1.33 ± 0.40 wildtype versus 2.2 ± 0.51 NPY-transgenic) and time (s) spend in the open arms were not significant (t (20) = 0.328; p = 0.747) between the NPY-transgenic and wildtype groups (mean ± SEM, 5.9 ± 1.2 wildtype versus 7.6 ± 1.35 NPY-transgenic) in the elevated plus maze. However, a pretest exposure in the form of one-hour physical restraint stress revealed significant differences in the open arm activity. A highly significant anxiogenic effect of the restraint was seen in the wildtype controls. The number of entries onto the open arms following restraint stress (mean ± SEM, 1.33 ± 0.40 no restraint versus 0.33 ± 0.14 restraint) is significant for wildtype rats (t (11) = 2.87; p = 0.0076; paired one-tailed t-test), but not significant for NPY-transgenic rats (t (9) = 0.583; p = 0.574; paired two-tailed t-test; mean ± SEM, 2.2 ± 0.51 no restraint versus 1.8 ± 0.36 restraint) when compared to the number of entries prior to restraint stress. The time (s) spent on the open arms following restraint stress (mean ± SEM, 5.9 ± 1.9 no restraint versus 2.2 ± 1.0 restraint) is significant for wildtype rats (t (11) = 1.84; p = 0.046; paired one-tailed t-test) but not significant for NPY-transgenic animals (t (9) = 0.729; p = 0.485; paired two-tailed t-test) when compared to non stress conditions (mean ± SEM, 7.6 ± 1.5 no restraint versus 6.6 ± 0.63 restraint). This effect was not due to non-specific changes in locomotor activity as the number of total entries onto any arm did not significantly differ between groups (p = 0.16) (*n* = 10 for aged NPY-transgenic rats and *n* = 12 for aged matched wildtype rats).

3.2. Open field

Results are expressed as means ± SEM of % entries (2A), % time spent in central area (2B), and total crossings (2C). Transgenic NPY rats displayed an increased preference for the central area of the open field (t (20) = 2.9; p = 0.007) % central entries; (mean ± SEM, 77.4 ± 3.8 NPY-transgenic versus 51.8 ± 7.1 wildtype). The % time in central area is also significant for the NPY-transgenic rats compared to wildtype rats (t (20) = 2.2; p = 0.04) % time; (mean ± SEM, 64.1 ± 2.9 NPY-transgenic versus 54.8 ± 3.2 wildtype). Locomotor activity, defined as total crossings in the open field, did not significantly differ between groups (t (20) = 0.19; p = 0.85) total crossings; (mean ± SEM, 176.3 ± 17.04 NPY-transgenic versus 182.3 ± 24.6.0 wildtype).

3.3. Morris water maze

The total escape latency per day did not significantly differ between groups (F (1, 60) = 1.1; p = 0.305) (Fig. 3A). There was no significant difference in the probe trial (t (20) = 1.488; p = 0.152) (Fig. 3B) and no significant difference in the visible trial (data not shown, p = 0.79) between aged NPY-transgenic rats and aged matched wildtype rats.

3.4. Delayed alternation version of the Morris water maze

Escape latency did not significantly differ between aged NPY-transgenic and aged wildtype animals ($F(2, 18) = 0.558$; d.f. = 2; $P = 0.582$, Fig. 4), nor did the delayed alternation performance per quadrant ($F(2, 18) = 1.226$; d.f. = 2; $P = 0.317$, data not shown). The effect per day as well as the effect per trial were however statistically significant for escape latency (respectively $F(8, 12) = 6.735$; d.f. = 8; $P = 0.002$ and $F(4,$

16) = 4.162; d.f. = 4; P = 0.017) but not the alternation performance per quadrant (respectively $F(8, 12)$ = 2.697; d.f. = 8; P = 0.059 and $F(4, 16)$ = 1.901; d.f. = 4; P = 0.159). The percentage of time spent in the quadrant containing the correct and the previous platform position was not significantly different between both groups ($F(2, 18)$ = 0.037; d.f. = 2; P = 0.964, data not shown). The effect per day as well as the effect per trial were however statistically significant (respectively $F(8, 12)$ = 8.835; d.f. = 8; P = 0.001 and $F(4, 16)$ = 3.501; d.f. = 4; P = 0.031). During the visual cued test, the percentage of time spent in quadrants containing the visible platform nor the quadrants previously containing platform positions was not significantly different between groups (visual cued test: P = 0.732; I: P = 0.732; IV: P = 0.882; I + IV: P = 0.778). During the probe test, the percentage of time spent in the quadrant previously containing the first platform position (I) was not significantly different between aged NPY-transgenic and aged wildtype animals (I: P = 0.484). However, wildtype animals spent significantly more time in the quadrant previously containing the second platform position (IV) (IV: P = 0.023). Swim speed neither during probe test nor during trials was significantly different between the two groups (P = 0.167 and P = 0.817, respectively).

3.5. Object recognition

The memory index (MI = $(Tn - to)/(Tn + to)$) did not significantly differ between the NPY-transgenic and wildtype animals (t (20) = 1.5; p = 0.147; mean ± SEM, 0.42 ± 0.05 NPY-transgenic versus 0.59 ± 0.09 wildtype) (Fig. 5). (n = 10 for aged NPY-transgenic rats and n = 12 for aged matched wildtype rats).

4. Discussion

The present study demonstrates that one-year-old transgenic rats NPY display marked anxiolytic behaviors, in agreement with earlier data reported in young transgenic rats [73] and various models of anxious behaviors [29,34]. However, and rather unexpectedly, the learning abilities of the aged NPY-transgenic rats were identical to aged-matched wildtypes in contrast to results obtained in younger animals in which memory deficits were noted in the transgenic cohort. It would thus appear that the anxiolytic-like properties of NPY are not age-sensitive in the transgenic rat model while learning disabilities observed early on in adulthood apparently mostly recover in one-year-old animals.

As in young rats overexpressing NPY, aged transgenic animals displayed anxiolytic-like behaviors in the elevated plus maze after a pretest stress exposure in the form of one-hour physical restraint. Baseline values of open and closed entries as well as time spent in each arm of the elevated plus maze did not differ significantly between the wildtype and NPY-transgenic groups before the stress challenge. As predicted, restraint stress significantly decreased exploratory behavior in the open arms (defined as the number of open arm entries and the time spend in the open arms) in the wildtype animals. However, there was no significant change in open arm activity following restraint stress in the NPY-transgenic rats. In addition, NPY-transgenic rats displayed anxiolytic-like behavior in a second animal model of anxiety, the open field. NPY-transgenic rats made more crossings, and spent more time in the central area of the open field. Locomotor activity (characterized as the number of total entries onto any arm in the elevated plus maze and total field crossings in the open field test) was similar between groups. The anxiolytic-

like effect of the NPY transgene in this cohort of aged rats is in agreement with data obtained in younger animals and supports the hypothesis that NPY may act to "buffer" behavioral effects of stress-promoting signals [29]. Indeed, NPY is consistently reported to elicit anxiolytic-like effects in diverse animal models of anxiety. For example, in rodents exposed to anxiety-provoking paradigms (i.e., elevated plus maze test, conflict test, and fear potentiated startle), exogenous NPY treatment produced behavioral responses similar to reference anxiolytic compounds such as the benzodiazepines [6–8,27].

The mechanistic action of NPY in anxiety related behaviors might involve central and/or peripheral mechanisms. For example, the overexpression of NPY peptide in the paraventricular, suprachiasmatic, supraoptic, and arcuate nucleus of the hypothalamus and in the hippocampus of NPY-transgenic rats may be potential neural substrates to the behavioral effects of the NPY transgene. The anterior and medial hypothalamus is involved in primary fear-generating circuits [68] and the paraventricular nuclei are important relays regulating hormonal and behavioral responses to stressors [4]. The limbic system, especially the amygdala and dorsal hippocampus, is strongly implicated in the formation and expression of emotional responses to stress [38,42,55,59].

A central mechanism involving the NPY Y_1 receptor subtype in the anxiolytic effect of NPY is supported by several pharmacological studies. For example, intracerebroventricular administration of an antisense oligonucleotide targeted at the Y_1 receptor messenger RNA (mRNA), attenuated NPY-induced anxiolytic-like effects [77]. In addition, the administration of the Y_1 antagonist BIBP3226 into the dorsal periaqueductal gray induced anxiogenic-like effects in the elevated plus maze [35]. Intra-

amygdalar injection of the Y_1 receptor selective antagonist, BIBO3304, blocked the anxiolytic-like properties of NPY in the social interaction test [62,63]. Hypothalamic NPY mRNA is readily unregulated by acute and repeated immobilization stress [39] although injection of the NPY Y_1 receptor antagonist BIBP3226 into the paraventricular nucleus of the hypothalamus was ineffective in modifying behavior in the elevated plus maze [35]. In the young NPY-overexpressing rats, there was a significant downregulation of the Y1 receptor subtype in the hippocampus (CA1, CA2, and dentate gyrus) but no significant change in corticosterone levels before or following a stress challenge [72]. This suggests a mechanism other than hypothalamic–pituitary–adrenal (HPA) axis modulation and supports the role of the limbic system in the modulation of anxiety behavior by NPY. The central role of the limbic system is also supported by a recent study that involves the Y_2 receptor subtype knockout mouse. Mice deficient in the Y_2 receptor subtype displayed an anxiolytic-like phenotype in the elevated plus maze and open field test [59]. These receptors are considered to be autoreceptors that provide negative feedback to NPY-ergic nerve terminals to modulate NPY release [12]. Y_2 receptor knockout mice are predicted to have increased endogenous peptide expression, analogous to the phenotype of NPY-transgenic rats that may contribute to the underlying mechanism responsible for anxiolytic-like behaviors modulated by NPY [59].

Although similar anxiolytic-like behaviors were observed in both young and old NPY-transgenic rats, there is a discrepancy between the effect of the NPY transgene on spatial learning and memory between the two groups. The performance by the young NPY-overexpressing rats in the Morris water maze (allocentric water maze test) revealed strong spatial learning/memory impairment. However, aged NPY-transgenic rats had no

such impairment (represented as identical escape latency and time spent in the platform quadrant during the probe trial) in the Morris water maze. Moreover, no significant difference was observed between aged NPY-transgenic and wildtype rats in delayed alternation performance (egocentric water maze test), which involves mainly the medial prefrontal cortex [15,52]. This indicates that the aged transgenic animals' ability to set up egocentric cognition maps has not been influenced by the expression of the NPY transgene. During the probe test wildtype animals spent significantly more time in the quadrant previously containing the second platform position. This might indicate that, although no alternation memory was acquired, wildtype animals remembered better the last position of the platform on the previous testing day. However, during the Morris water maze test, no significant difference in escape latency between wildtype and transgenic animals was observed. All transgenic and wildtype animals tested in both the allocentric and egocentric water maze test, showed normal escape latencies and swim speeds under visible platform conditions, demonstrating that neither the motivation nor the ability to find the platform was affected by the transgene. Furthermore, both groups showed no significant differences in non-spatial learning/memory. This was calculated as a memory index and defined as the time spent exploring a novel versus familiar object in the one trial object recognition task. These results suggest that learning and memory processes are unaffected in aged NPY-transgenic rats.

It is unclear why the deficit in spatial memory was not observed in older NPY-transgenic rats as it was in the younger animals. However, there is a progressive decrease of NPY peptide concentration in the rat frontal cortex, paraventricular nucleus and in the dentate gyrus of the hippocampus as the animal ages [30,74]. Similarly, human studies

have revealed a significant correlation between the decline in plasma NPY levels and increasing age [11]. NPY mRNA increases have been reported in some areas of the rat brain such as striatum and medulla oblongata [31]. The lower content of NPY in neuronal cells suggests that the amount of NPY released in aged rats would be lower than in young rats [26]. Interestingly, low doses of NPY have been shown to enhance working memory while high doses of NPY have had the opposite effect [71]. Consequently, higher levels of the NPY transgene in younger animals may have had an analogous effect to a high dose of NPY. In older animals, the NPY transgene may have had the reverse effect. Another possibility may be related to intrinsic changes that occur during the aging process and the effect of the NPY transgene at different stages of the animal's life.

Age-related changes in L-type channel activation [21] may be involved in the apparent contradictory results obtained in spatial memory between young and aged NPY-transgenic rats. Calcium currents through L-VSCC are increased in the CA1 of the hippocampus in aged rats [75]. It has been proposed that age related deficits in cognitive processing involve dysfunction of hippocampal synaptic plasticity, especially long term potentiation (LTP). LTP is a proposed candidate for the cellular mechanism of memory that may be affected during aging. Interestingly, L-VSCC dependent LTP is increased in aged rats [65] and patients with Alzheimer's disease have shown increased L-VSCC protein expression in the hippocampus [14]. The degree of current through the L-VSCC has been correlated with the degree of learning impairment in a hippocampal dependent task [69] and chronic treatment with the L-VSCC antagonist nimodipine ameliorates age-related working memory deficits to improve learning in the Morris water maze

[41,64,75]. Consequently, excess calcium influx through L-VSCC may be detrimental to memory formation in aged animals.

Recently, it was shown that the inhibitory effect of NPY induced by Y_1 or Y_2 receptors in intracellular calcium changes is mainly due to the inhibition of N- and L-type channels in the adult rat hippocampus [67]. Consequently, the deficit in spatial memory observed in young NPY-overexpressing rats is in agreement with in vitro studies as NPY within the hippocampal formation reduces presynaptic calcium entry at the Schaffer collateral-CA1 synapse and suppresses the formation of LTP in the dentate gyrus [13,56,78]. However, in aged NPY-transgenic rats, the ability of NPY to inhibit L-type calcium channels within the hippocampus [66] may have had a beneficial impact on learning and memory processing. It would be of interest to directly investigate this hypothesis in future studies.

In conclusion, the anxiolytic-like profile observed in young NPY-transgenic rats is maintained in older animals, thus providing support for a role of NPY and related peptides in anxious behaviors. This is apparently not the case for the cognitive deficits noted in young but not one-year old animals. These results also provide additional evidence for the mechanistic dissociation between anxiety and cognition-related behaviors modulated by NPY and its receptors.

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Figure Legends

Figure 1: Elevated plus maze. The percentage of open arm entries and the percentage of time spend in the open arms is expressed graphically as % open (open/(open + closed)) (Fig. 1). Baseline values for the percentage of open entries ($t(20) = 0.45$; $p = 0.66$) and percentage of time spend in the open arms were not significant ($t(20) = 2.04$; $p = 0.84$) between the NPY-transgenic and wildtype groups. However, a pretest exposure in the form of one-hour physical restraint stress revealed significant differences in the open arm activity. A highly significant anxiogenic effect of the restraint was seen in the wildtype controls. The percentage of entries onto the open arms following restraint stress is significant for wildtype rats ($t(11) = 3.92$; $p = 0.0024$), but not significant for NPY-transgenic rats ($t(9) = 0.33$; $p = 0.75$) when compared to the percentage of entries prior to restraint stress. The percentage of time spent on the open arms following restraint stress is significant for wildtype rats ($t(11) = 2.33$; $P = 0.039$) but the percentage is not significant for NPY-transgenic animals ($t(9) = 0.259$; $p = 0.80$) when compared to non-stress conditions. * $p < 0.05$; ** $p < 0.01$ ($n = 10$ for aged NPY-transgenic rats and $n = 12$ for aged matched wildtype rats).

Table 1: Total arm activity in elevated plus maze for NPY-transgenic rats and control wildtype rats in the elevated plus maze in stress and non-stress conditions. Data in presented as # of open and closed entries and time (s) for each condition. (n=10 for aged NPY-transgenic rats and n=12 for aged matched wildtype rats).

Figure 2: Open field. The behavioral profile of NPY-transgenic rats and control wildtypes in the open field. NPY-transgenic rats made significantly more entries into the

central area (A) (t (20) = 2.9; p = 0.007) and spent more time in the central area (B) (t (20) = 2.2; p = 0.04). Locomotor activity (C, described as the total number of crossings on the open field) is not significant between groups (t (20) = 0.19; p = 0.85). Values are ± SEM; * $p < 0.05$ (n = 10 for aged NPY-transgenic rats and n = 12 for aged matched wildtype rats).

Figure 3: Escape latency in the Morris water maze. Escape latency in the Morris water maze did not significantly differ between NPY-transgenic and wildtype animals (A) F (1,60) = 1.1; p = 0.305. Moreover, the amount of time spent in the probe quadrant during the probe trial in the Morris water maze was not significant between groups (B) t (20) = 1.488; p = 0.152. ANOVA repeated measures. Values are ± SEM for total escape latency and time spend in probe quadrant for both groups of animals (n = 10 for aged NPY-transgenic rats and n = 12 for aged matched wildtype rats).

Figure 4: Delayed alternation version of the Morris water maze. Delayed alternation performance was not significantly different between aged NPY-transgenic and aged wildtype animals ($F(2, 18)$ = 0.558; d.f. = 2; P = 0.582) ANOVA repeated measures. Values are ± SEM (n = 9 for aged NPY-transgenic rats and n = 12 for age matched wildtype rats).

Figure 5: Object recognition. Behavioral profile of NPY-transgenic rats and control wildtypes in the object recognition test. Results are expressed as mean ± SEM for the memory index (MI = $(tn - to)/(tn + to)$) of each animal. The mean index did not significantly differ between the NPY-transgenic and wildtype animals (t (20) = 1.5; p = 0.147). (n = 10 for aged NPY-transgenic rats and n = 12 for aged matched wildtype rats).

Figure 1: Elevated Plus Maze

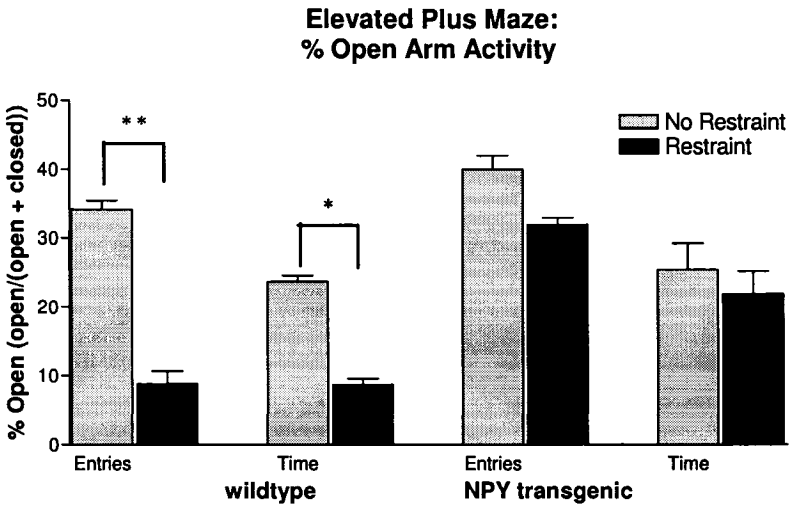


Table 1: Total arm activity in the elevated plus maze

	Wildtype				NPY Transgenic			
	Entries #		Time (s)		Entries #		Time (s)	
Arm	No Restraint	Restraint	No Restraint	Restraint	No Restraint	Restraint	No Restraint	Restraint
Open	16	4	71.1	26.1	22	18	76.3	66.3
Closed	30	41	228.9	273.9	33	31	223.7	233.7
Total	46	45	300	300	55	49	300	300

Figure 2: Open Field

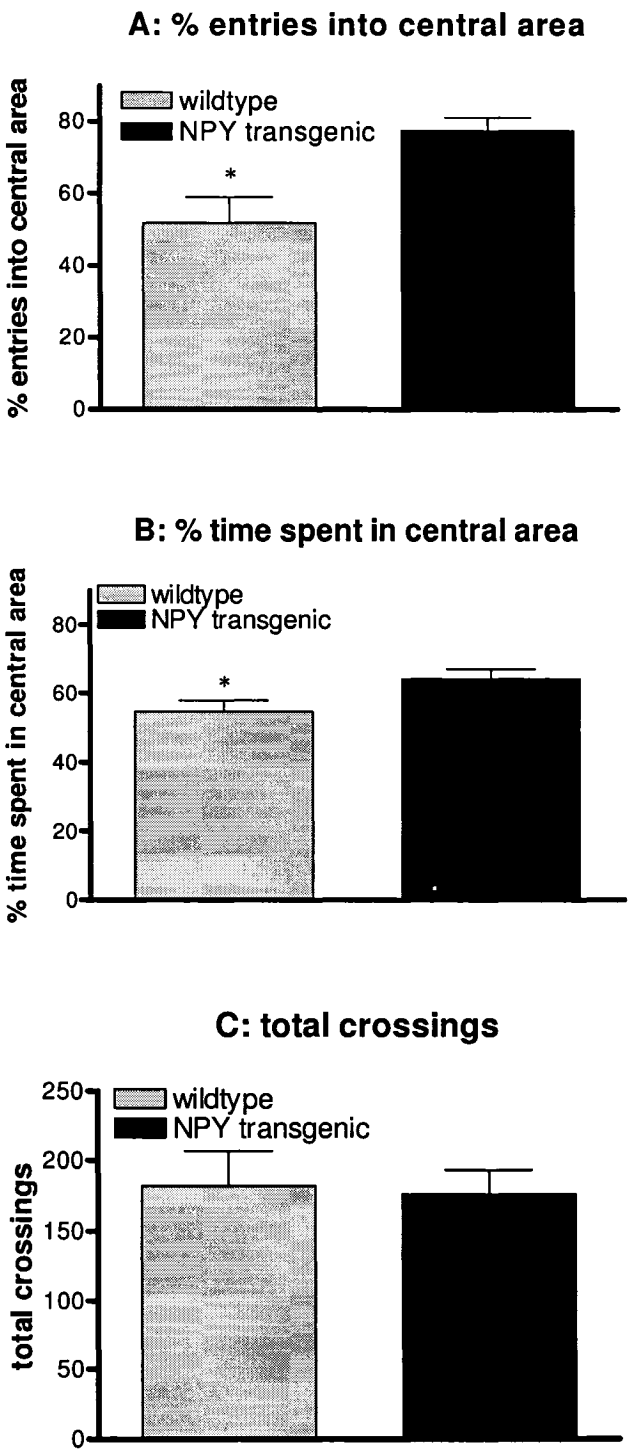
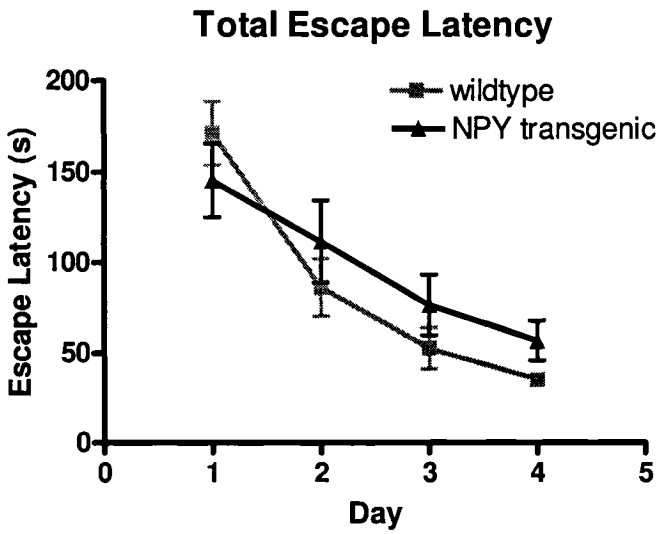


Figure 3: Morris Water Maze



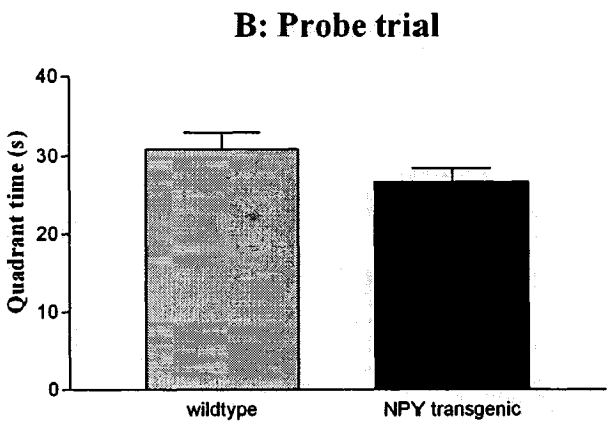


Figure 4: Delayed alternation version of Morris water maze

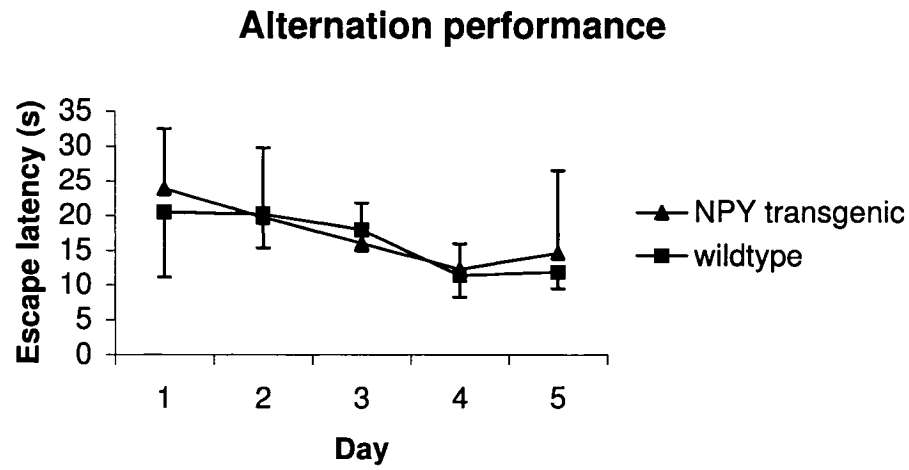
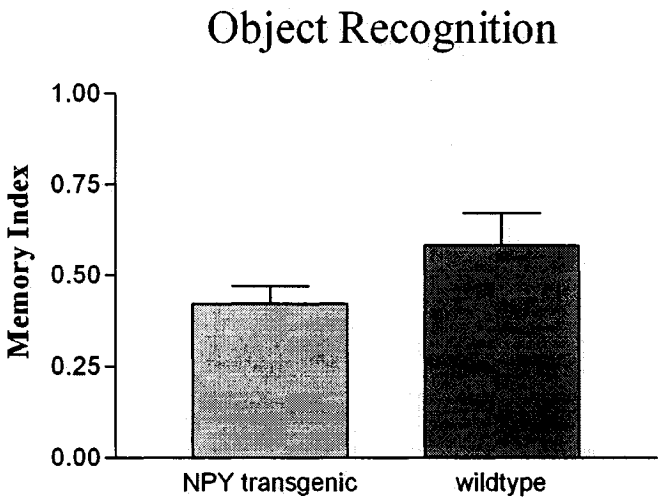


Figure 5: Object recognition



Appendix B: Ethics Certificates

McGill

University Animal Care Committee

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Tel.: (514) 398-2837
Fax: (514) 398-4853
www.mcgill.ca/rgo/animal

October 14, 2003

The McGill University Animal Care Committee certifies that
Cristina Carvajal has successfully completed a
Rat Methodology Workshop on ***September 25, 2003***.

The training included the following procedures:

- ✔ Handling and restraint
- ✔ Injections: subcutaneous, intramuscular, intraperitoneal, intravenous *
- ✔ Gavage (tube feeding)
- ✔ Blood collection: saphenous and cardiac puncture
- ✔ Determination of anaesthetic depth
- ✔ Euthanasia by cervical dislocation
- ✔ Euthanasia by decapitation

** Intravenous injection has only been demonstrated, for certification of this procedure, a special session is needed*

Certification is valid for 5 years, starting on the date of the workshop.

Suzanne Smith
Research Ethics Officer for Animal Studies
animalcare@mcgill.ca

(Confirmation of training can be obtained by request to the above email address)

Note: Trainee must keep this certificate as other institutions may request it as evidence of training

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October 14, 2003

The McGill University Animal Care Committee certifies that
Cristina Carvajal has successfully completed a
Mouse Methodology Workshop on ***October 2, 2003***.

The training included the following procedures:

- ✔ Handling and restraint
- ✔ Injections: subcutaneous, intramuscular, intraperitoneal, intravenous *
- ✔ Gavage (tube feeding)
- ✔ Blood collection: saphenous and cardiac puncture
- ✔ Determination of anaesthetic depth
- ✔ Euthanasia by cervical dislocation

** Intravenous injection has only been demonstrated, for certification of this procedure, a special session is needed*

Certification is valid for 5 years, starting on the date of the workshop.

Suzanne Smith
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Note: Trainee must keep this certificate as other institutions may request it as evidence of training

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July 21, 2004

The McGill University Animal Care Committee certifies that
Cristina Carvajal has successfully completed the

Advanced Level
of the
Theory Training Course on Animal Use for Research and Teaching
on
June 23, 2004.

The training includes the following topics:

- **Basic Level:** Regulations & Procedures, Ethics, Basic Animal Care, Occupational Health & Safety
- **Advanced Level:** Anesthesia, Analgesia, Euthanasia, Categories, Influencing Factors, and Environmental Enrichment

Please note that this certificate does NOT include practical training, which is obtained by successfully completing an Animal Methodology Workshop where another certificate is issued.

Certification is valid for 5 years, starting on the date indicated above.

Deanna Collin
Animal Care Training Coordinator, animalcare@mcgill.ca

(Confirmation of training can be obtained by request to the above email address)

Note: Trainee must keep this certificate as other institutions may request it as evidence of training

Appendix C: Copyright

ıy 2005

Our ref: HG/HDN/MAY05/J094

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Ms Carvajal

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