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Your Brain on Fat:
The Role of Diet in Depression Like Behaviours

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In
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This thesis is dedicated to my Dad for the inspiration and David for all the support...

Αυτή η εργασία είναι αφιερωμένη στον μπαμπά μου για την έμπνευση και στον David για την υποστήριξη όλο αυτό το διάστημα...
Abstract

Epidemiological and clinical studies indicate that overweight and obesity are associated with increased risk for depression, but the mechanisms linking dietary components with the development of depression phenotype are poorly understood. To investigate this relationship, we utilized lipidomic and transcriptomic methods to evaluate the effects of diet on the brain. We identified a molecular mechanism by which even short exposure to high-fat diet (HFD) alters hypothalamic functions leading to depression. Consumption of a HFD induced in the hypothalamus accumulation of different saturated and unsaturated fatty acids, suppression of the 3’, 5’-cyclic AMP (cAMP)/protein kinase A (PKA) signaling pathway, and increase the RNA levels of the free-fatty acid receptor 1 (FFAR1), a G protein-coupled receptor (GPCR). Palmitic acid, but not oleic acid, both FFAR1 ligands, suppressed PKA activity in a neuronal cell line that expresses high levels of FFAR1. Deficiency of phosphodiesterase 4A (PDE4A), an enzyme that degrades cAMP and modulates GPCR signalling, protected from dietary and genetic induced depression phenotype in mice. These findings demonstrate that specific dietary fatty acids can enter the brain to disrupt hypothalamic functions by suppressing cAMP/PKA signaling possibly via the activation of FFAR1/PDE4A. FFAR1 inhibition and increase of cAMP signaling in the hypothalamus may offer a novel strategy to counteract the effects of diet on depression.
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Author’s Declaration

I hereby declare that, the work presented within this thesis has been performed by myself, unless otherwise cited or acknowledged. The work is entirely of my own composition and has not been submitted, in whole or in part, for any other degree or professional qualification at the University of Glasgow or any other institution.

Eirini Vagena
September 2014
Definitions/Abbreviations

adenylyl cyclase AC
agouti-related protein agRP
A-kinase anchoring protein AKAP
AMP-activated protein kinase AMPK
Arachidic acid AA
arcuate nuclei ARC
blood brain barrier BBB
Body mass index BMI
Brain derived neurotrophin factor BDNF
cAMP responsive element binding protein CREB
central nervous system CNS
coactivator of CREB cbp
cyclic AMP cAMP
Dietary induced obesity DIO
docosahexaenoic acid DHA
eicosapentaenoic acid EPA
Electroconvulsive therapy ECT
elevated plus maze EPM
Epididymal fat IP
fatty acid FA
fluorescence resonance energy transfer FRET
Food and Drug Administration FDA
forced swim test FST
free fatty acid FFA
free fatty acid glucose-stimulated insulin secretion GSIS
functional magnetic resonance imaging fMRI
G protein-coupled receptor kinase GRK
Genetically induced obesity GIO
-glucose dependent insulin stimulation GDIS
-glucose tolerance test GTT
G-protein coupled receptor GPCR
high fat diet HFD
High throughput screening HTS
Hypothalamus pituitary adrenocortical HPA
Inflammatory response system IRS
ingenol 3-angelate I3A
inositol triphosphate IP3
insulin tolerance test ITT
intracerebrovascular i.c.v.
intraperitoneal i.p.
knock out KO
lateral hypothalamic area LHA
linker region LR
Long chain PUFAs (LC-PUFAs) LC-PUFAs
major depressive disorder MDD
<table>
<thead>
<tr>
<th>Term</th>
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<tr>
<td>MAP KINASE 2</td>
<td>MK2</td>
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<tr>
<td>Melanin-concentrating hormone</td>
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Chapter 1

Introduction
Chapter 1  

Introduction

Depression is a devastating mental disorder which affects more than 340 million people globally according to the world health organization (WHO, 2012). By the year 2030 major depression is expected to be one of the three leading causes of morbidity in developed countries (Hughes et al., 2011; Murray and Lopez, 1997). There are several risk factors for depression; one of them is obesity (Roberts et al., 2003; Roberts et al., 2000). Obesity is a major public health concern with alarmingly increasing rates in the western modern societies. The prevalence of obesity has doubled the last 50 years. Given the increasing rates of obesity and their positive correlation with depression, even a small increase of obesity might lead to threatening levels of mental disorders for the society. Dietary and lifestyle changes over the last 3 decades are the major reason for the dramatic increase of these two chronic human diseases that are also associated with various other health complications (Compton et al., 2006). Given the increase in obesity and depression in western societies, identifying the underlying molecular links may provide important diagnostic and therapeutic opportunities.

1.1. Obesity

Obesity is a metabolic disorder reaching epidemic proportions; is a public health issue and a growing problem (Taubes, 1998; Popkin and Doak, 1998). Obesity develops as the result of energy imbalance when energy intake exceeds energy expenditure and characterized by excess of fat deposition in the body. Western societies are based on high caloric food consumption characterized by high fat, sugars and salt but low in vitamins, minerals and other micronutrients. Excess of caloric intake from fat and refined carbohydrates combined with the modern sedentary western lifestyle characterized by physical inactivity is the main reason for appearance of the epidemic of obesity for millions of people worldwide (WHO 2012; Venables and Jeukendrup, 2009). Obesity is measured by the body mass index (BMI); it is defined as a person’s weight in kilograms divided by the square of his height in meters (kg/m²) BMI of 25 kg/m² or greater describes overweight, while BMI of 30 kg/m² or greater describes obese in the general population. The abnormal or excessive fat accumulation in the overweight and obese individuals is mainly responsible for their impair health.
There are two main consequences of obesity: metabolic syndrome and increased risk for various diseases. Obesity confers a profound association with various health complications such as hypertension, dyslipidemia, diabetes mellitus, coronary heart disease, atherosclerosis, stroke, as well as cancer and increased mortality (NIH 1998; Allison et al., 1999a; 2000; Kannel et al., 1967; Poirier et al., 2006; Das, 2010). Even though obesity has been linked with various diseases not commonly associated with metabolic syndrome, such as stroke it has never been shown to be a causative factor for any of them except a study recently published revealing that obesity is a causative factor for the development of hepatic cancer (Park et al., 2010b).

1.2. Depression

Depression is one of the most common and a serious affective illness that can affect people at any age from early childhood to late life and has a great socioeconomic cost (Wittchen, 2012). It is characterized by a state of low mood and aversion to activity and can affect a person's thoughts, behaviour, feelings and physical well-being. The development and progression of depression is influenced by genetic, epigenetic and environmental factors. However, depression most commonly occurs during adulthood, which is the most productive years of one’s life, and this can exert considerable cost upon the society and the individual. Depression is one of the most common psychiatric disorders that patients are looking for outpatient care (Kimerling et al., 1999). The overall risk of developing psychiatric disorders such as depression typically rises during the adolescent years (Brooks et al., 2002; Sorenson et al., 1991) and is associated with future depression (Lewinsohn et al., 1999). Depression is associated with increased risk of coronary heart disease, stroke, myocardial infarction, heart failure, low bone mineral density and increased mortality (Keller, 2008).

There are two different ways to assess depression: either the Montgomery-Asberg Depression Rating Scale (MADRS) (Montgomery and Asberg, 1979) or the 17-item Hamilton Depression Rating Scale (HAMD-17) (Hamilton, 1960). The symptoms of depression can vary considerably and even within the same group
of depressed patients there can be major differences with diverse symptoms. However, persistent depressed mood and anhedonia are the two core criteria for depression. Depression is a widespread and debilitating disorder whose pathophysiology remains enigmatic although abnormalities in monoamine signalling have been implicated (Charney, 1998). Indeed, some of the major drugs developed for depression target the monoamine signalling (Shelton, 2007).

1.2.1. Physiological pathways altered in depressed patients

Depression has predominantly been linked with altered levels of brain neurotransmitters (Charney, 1998), such as acetylcholine (enhances memory) (Higley and Picciotto, 2014), serotonin (regulates sleep, appetite, mood) (Albert et al., 2014), norepinephrine (Shelton, 2007), dopamine (Moraga-Amaro et al., 2014), glutamate (play a role in schizophrenia and bipolar disorder) (Sanacora et al., 2004; Burnouf et al., 2013) and GABA (Sanacora et al., 2004; Luscher et al., 2011; Fatemi et al., 2011). However, there are many other physiological pathways that are affected in depression (Heninger et al., 1996).

Stress is a major vulnerability factor for depression and depressive disorders have been well known to associate with stress disorders (Kendler et al., 1999; Southwick and Charney, 2012). The main pathway involved in stress response is the hypothalamic-pituitary-adrenal (HPA) pathway. Depression is accompanied with excessive activity of the HPA axis and dysregulation of glucocorticoid release in response to stressful experience that are associated with depression (Holsboer and Ising, 2010; McEwen, 2007).

Furthermore, depression is associated with increased inflammatory response and cytokine production due to activation of the immune system (Schiepers et al., 2005). Cytokines are a group of polypeptide mediators that are associated with the regulation of immunity and inflammation as well as the regulation of the HPA axis (Turnbull and Rivier, 1995). Cytokines such as TNF-α and IL-1β can lower the availability of tryptophan, one of the precursors of neurotransmitters, alter the neurotransmitter metabolism as well as the mRNA of the neurotransporters (Watkins et al., 2014; Logan, 2003). Inhibition of TNF-α, a
cytokine that is elevated in the adipose tissue of obese rodents, improves glucose tolerance and insulin sensitivity, which are main characteristics of obesity (Hotamisligil et al., 1993) as well as reducing the depression phenotype in mice (Raison et al., 2006). In agreement to the hypothesis that increased cytokine production leads to depression is the fact that systemic and chronic inflammation is accompanied by increased cytokine production (Dantzer et al., 2008). Moreover, obesity is associated with chronic and low grade inflammation (Weisberg et al., 2003; Xu et al., 2003; Hotamisligil, 2010). This can activate the immune system, which, in turn, can alter brain functioning to induce a depression phenotype (Dantzer et al., 2008).

Depression is also associated with a distinct pattern of cognitive impairment, including memory deficits, which are a core element of depression (Arendash et al., 2001). Impairments in cognitive performance in depressed patients have been identified across a number of cognitive domains including executive functions (Baudic et al., 2004; Merriam et al., 1999; Nebes et al., 2003), working memory (Christopher and MacDonald, 2005), as well as emotional memory (Siegle et al., 2007). Moreover, evidence suggests a positive correlation between the severity of depression symptoms with cognitive impairments (Austin et al., 2001; McDermott and Ebmeier, 2009). Different neuronal mechanisms have been proposed to recapitulate the cognitive model of depression, however the neurobiological underpinnings of cognitive deficits in depression remain poorly understood (Disner et al., 2011). In accordance to the human studies, the socially defeated mouse model, which is an animal model of depression, exhibits cognitive and neuronal impairments that correlate with the depression phenotype (Yu et al., 2011).

1.3. Obesity is linked with depression

Depression is a debilitating mental illness that is often comorbid with metabolic abnormalities such as central obesity and impaired glucose tolerance which are also associated with obesity (Weber et al., 2000). Many metabolic pathways have linked obesity with depression (Hryhorczuk et al., 2013) and there is a clear interconnectivity between them. Cytokines such as TNF-α and IL-1β can also modulate the HPA axis causing resistance to glucocorticoid hormones that can
lead to obesity or depression (Turnbull and Rivier, 1995). Activation of the sympatoadrenal system and production of pro-inflammatory cytokines caused by depression can induce insulin resistance and contributes to obesity (Makki et al., 2013). Moreover, depression is accompanied by deleterious changes in the autonomic system, activity levels, reproductive system and lipid metabolism all of which have also been associated with the pathophysiology of obesity (Allison et al., 1999a; 2000; Kannel et al., 1967; Poirier et al., 2006). Increased levels of proinflammatory cytokines observed in obesity may interfere with neurotransmitter metabolism, alter neurotransmitter RNA expression levels and inhibit brain derived neurotrophin factor (BDNF) expression a major protein involved in depression as discussed below (Hayley et al., 2005; Anisman, 2009).

A major challenge in exploring the relationship between obesity and depression in human studies is the controlling for dietary, environmental and lifestyle factors. Among patients with depression and obesity the heterogeneities in lifestyles and diets make it difficult to study a correlation or causative relationship as both disorders are highly associated with other lifestyle traces such as physical inability or eating disorders. Due to the above reasons, identifying the strength of correlation between obesity and depression has proven difficult and would require large cohorts of individuals while controlling for factors such as exercise or lifestyle.

Epidemiological and clinical studies have been effective for the identification of the link between obesity and depression (Zhong et al., 2010; Leonore de Wit and Frans Zitman, 2010; Boutelle et al., 2010; Stunkard et al., 2003; Simon et al., 2010; McElroy et al., 2004; Faith et al., 2002; Luppino et al., 2010; Palinkas et al., 1996; Roberts et al., 2003; Dong et al., 2004; Roberts et al., 2002; Stunkard, 1957). Both these disorders incur substantial costs to society (Allison et al., 1999b; Druss et al., 2000) and the individual (Wells et al., 1989). However, most of these studies typically lack large, representative samples and did not employ validated measures of psychopathology.

The positive association between obesity and depression is higher between women than men. It is more common for women with high BMI to develop depression compare to men (Istvan et al., 1992; Carpenter et al., 2000; Onyike
et al., 2003) suggesting sex differences play a role for that link. It has been suggested that women not only have a greater biological and psychological vulnerability to depression than do men but they are also more likely to experience acute and chronic stressors that would activate a predisposition to depression (Nolen-Hoeksema and Ahrens, 2002).

Certain studies have shown that obesity, defined as a pathologically elevated BMI, can predict the development of major depressive disorder (Roberts et al., 2003; Roberts et al., 2000; Carpenter et al., 2000). Interestingly, it has been shown that a short intervention for 4 weeks of adoption of an obesity provoking behaviour decreases the health related quality of life and induced a depression phenotype (Ernersson et al., 2010). That effect was temporary when followed up for 6 to 12 months after the short intervention and there was no remaining influence (Ernersson et al., 2010). On the other hand, it has also been shown that depression can promote the development of visceral obesity in humans (Needham et al., 2010) and perturbed lipid metabolism, a common pathological characteristic in obesity that has also been observed in patients with depression (Rudisch and Nemeroff, 2003). Baseline depression significantly increased the probability for developing obesity over time (Lupino et al., 2010; Pan et al., 2012). Major depressive disorder among young children can predict a greater body mass index in adult life (Pine et al., 2001). Another interesting point that links together the pathways of obesity and depression is that the majority of currently prescribed psychotropics, which includes antipsychotics, antidepressants, and mood stabilizers, will generate weight gain and ultimately obesity in some patients over the course of clinical treatment (Schwartz et al., 2004). Whether this increase is because of the direct effect of the drug or because of the mood change of the individual is unknown. Moreover, this suggests that antidepressant treatment might differentially regulate the pathways of depression and body weight gain as in the short term it reduces the depression phenotype but increases body weight. However, whether this body weight gain during antidepressant treatment can increase the chances of the development of depression later in life needs further investigation.

Even though a correlation between obesity and depression has been proposed in different clinical and epidemiological studies this correlation is stronger
between extreme obesity and depression phenotype. The most obese individuals are at increased risk for the development of depression (Onyike et al., 2003). Being obese but not overweight during adolescence has been shown to be a predictor of depression later in life (Boutelle et al., 2010). The extremely obese individuals that sought bariatric surgery were found to have higher depression scores than the non-extremely obese (Berkowitz and Fabricatore, 2005). Due to the worldwide progressive increase of the obese population a rapid increase of depression will have a major socioeconomic effect. The extremely obese people also have greater impairments in health-related life quality than do their less obese peers and this might also be influencing their increased risk for depression. However, the precise mechanism underlying the interaction between obesity and depression remains to be elucidated.

Interestingly, obesity has also been described with impaired noradrenaline signalling in specific organs (Levin and Dunn-Meynell, 2000), a main signalling pathway affected in depression. Altered weight gain patterns have been linked to changes in the expression of β-adrenergic receptors (Charon et al., 1995; Raasmaja and York, 1988) and in patients chronically treated with β-adrenergic blockers, one of the major side effects is a prominent weight gain (Sharma et al., 2001). Additionally genetically modified mice that lack all β-adrenergic receptors become massively obese when fed a HFD (Bachman et al., 2002).

1.4. Current antidepressant treatments for depression

50 years ago, antidepressants were discovered by serendipity as some treatments for tuberculosis, were exerting a beneficial effect in the sense of well-being (Selikoff and Robitzek, 1952; Bloch et al., 1954). This observation set iproniazid as the first antidepressant drug (Loomer et al., 1957). The range of different molecular pathologies that can underpin depression and no doubt complex biochemical phenotypes in patients with depression has made it hard to find drugs that treat all forms of depression. Therefore it has been very difficult to find an antidepressant treatment that will satisfy all patients and minimize the occurrence of side effects. Rather we can expect advances to be made when biomarkers allow the particular cause of individual patients to be uncovered and direct them to the most effective therapies. For that reason different
antidepressant treatments have been established for the different forms of depression that are discussed below. Invasive and non-invasive antidepressant treatments have been developed with very promising data coming from the non-invasive brain stimulations.

There are two main categories for the antidepressant treatment; the chemical and non-chemical treatments. The non-chemical treatments of depression include electroconvulsive therapy, vagus nerve stimulation and repetitive transcranial magnetic stimulation. The chemical treatments are invariably based on the monoamine hypothesis of depression that states that depression is characterized by reduced levels of the monoamines, such as noradrenaline and serotonin, in the brain (Nestler et al., 2002a). Intense attention has been focused on the targeting of monoamines (Hamon and Blier, 2013), particularly norepinephrine, epinephrine and dopamine for the treatment of depression over the last 40 years due to this theory (Bunney and Davis, 1965; Schildkraut, 1995; Coppen, 1967). The neurotransmitter system has been implicated in the actions of many antidepressant drugs (Frazer and Conway, 1984; Charney, 1998). Drugs that can reverse the reduction of the monoamines or slow down their metabolism in synapses in order to block the inactivation of the brain serotonin and noradrenaline pathways are proposed to have a potential antidepressant action. However, the monoamine hypothesis cannot explain the full aetiology and pathogenesis of depression (Heninger et al., 1996; Hyman and Nestler, 1996; Nestler, 1998; Nestler et al., 2002b).

1.4.1. Categories of antidepressant drugs

The neurotransmitter’s elevated concentration in the brain is exerted by antidepressants either by acute inhibition of its reuptake or of monoamine oxidase activity (Banerjee et al., 1977; Wolfe et al., 1978). Therefore, the two major classes of the medicine category of antidepressants are the monoamine reuptake inhibitors that prevent the normal recapture of either serotonin or noradrenaline by their transport back into the presynaptic nerve-terminal as well as the monoamine oxidase inhibitors that both lead to an increase or serotonin or noradrenaline in the synapse. Table 1.1 summarizes the main antidepressant categories with the main drugs used in each category.
The most commonly prescribed class of antidepressants are those that act by specifically preventing the uptake from the presynaptic neuronal cell of serotonin therefore upregulating the amount of serotonin in the synapse and called selective serotonin reuptake inhibitors (SSRIs). This results in the postsynaptic cell to be constantly activated by the serotonin in the synapse. Serotonin is synthesized in cell bodies of the brain stem raphe nuclei. The therapeutic response of SSRIs occurs after 3-4 weeks of treatment (Belmaker and Agam, 2008). SSRIs are often the first line of treatment for depression due to their general safety and few side effects. Fluoxetine (Prozac) is a well-known antidepressant that belongs in the SSRI (Wong et al., 2005). One of the potential actions of fluoxetine is speculated to be the increase neuronal cell proliferation in the dentate gyrus (Manev et al., 2001). Monoamine oxidase inhibitors (MAOIs) is another major class of antidepressants that slows down the normal enzymatic degradation of these neurotransmitters (Schwartz, 2013). Monoamine oxidase is the major enzyme that breaks down serotonin or noradrenaline therefore when it is inhibited there is an increase of the monoamines in the synapse. Iproniazid was the first antidepressant (Loomer et al., 1957) and the first member of the MAOIs family (Zeller and Barsky, 1952). MAOIs have been shown to impair the breakdown of tyramine a substance in food such as aged cheese, wines, most nuts and chocolate (Gray and Gray, 1989). High blood concentration of tyramine due to consumption and administration of MAOIs can elevate blood pressure dangerously. Therefore MAOIs are prescribed only after the failure of other first line treatments due to these potentially serious drug and food interactions and then only with close dietary supervision.

Tricyclic or tetracyclic antidepressants consist of either three or four chemical rings. Their therapeutic action is speculated to occur by increasing the level of norepinephrine and serotonin in the synapses, however the exact molecular mechanism remains unknown (Feighner, 1999). They are used to treat moderate to severe depression. Tricyclic antidepressants have a cardiotoxic potential, and if taken overdose they can cause life threatening heart-rhythm aberration (Thanacoody and Thomas, 2005) and that's the reason why they are not the first option for antidepressant treatment.
Drugs that are effective in treating depression and cannot be classified in one of the previous categories are called atypical antidepressants. Their antidepressant action happens by increasing the levels of neurotransmitters in synapses.

Mood stabilizers, stimulants and antipsychotic drugs are also used to treat depression. Antipsychotic drugs in combination with antidepressants have been found to be effective mood stabilizers and are therefore used to treat bipolar depression. Stimulants are commonly used in combination with other antidepressants or medication for the treatment of depression that is resistant to other medications but they are rarely used alone due to their potentially addictive nature.

Transcranial direct current stimulation (tDCS) trials for major depressive disorder (MDD) have shown positive but mixed results therefore further studies should be performed (Brunoni et al., 2013). Combination of the pharmacological and non-pharmacological treatments with deep brain stimulation appears to be working better than using just a single therapy (Brunoni et al., 2013).

Electroconvulsive therapy (ECT) is a non-chemical therapy that is reserved for severely depressed patients or patients that are at high risk for suicide that do not respond to conventional drug therapy or other antidepressant treatments do not provide a satisfactory relief from the symptoms (Medda et al., 2014). During the ECT procedure an electric current is passed through the brain to produce controlled convulsions (seizures) which is speculated that it works by a massive release of neurotransmitters in the brain (Faedda et al., 2010).

All the previous classes of medications that treat depression (MAOIS, SSRIS, TCAS and atypical antidepressants) have shown to have an effect on norepinephrine and serotonin as well as other neurotransmitters that are thought to be involved in depression (Dell’Osso et al., 2011). Some newer developed antidepressant drugs called dual-action appear to have a particular robust effect on both the norepinephrine and serotonin systems (Duman and Voleti, 2012). However, the various medications affect the different neurotransmitters in varying degrees. These medications seem to be very effective for the more severe and chronic cases of depression. For example, Effexor is a serotonin reuptake inhibitor that
at lower doses, shares many of the safety and low side-effect characteristics of the SSRIs (Sansone and Sansone, 2014). At a higher dose though it appears to block the reuptake of norepinephrine as well (Sansone and Sansone, 2014). In accordance to that, Cymbalta and Pristiq tend to act as equally powerful serotonin and norepinephrine reuptake inhibitors regardless of the dose (Sansone and Sansone, 2014).
Table 1.1. Main categories of antidepressant treatment and their representative drugs. A summary of the main antidepressant categories with the main drugs used in each category and their commercial names.

<table>
<thead>
<tr>
<th>Category</th>
<th>Drugs</th>
</tr>
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| Selective serotonin reuptake inhibitors (SSRIs) | fluoxetine (Prozac)  
paroxetine (Paxil)  
sertraline (Zoloft)  
citalopram (Celexa)  
fluvoxamine (Luvox)  
escitalopram (Lexapro) |
| Monoamine oxidase inhibitors (MOAIs)          | phenelzine (Nardil)  
tranylcypromine (Parnate)  
Isocarboxazid (Marplan) |
| Tricyclic antidepressants (TCAs)              | amitriptyline (Elavil)  
protriptyline (Vivactil)  
desipramine (Norpramin)  
nortriptyline (Aventyl, Pamelor)  
imipramine (Tofranil)  
trimipramine (Surmontil)  
perphenazine (Triavil)  
amoxapine (Asendin)  
Clomipramine (Anafranil)  
Doxepin (Sinequan)  
Maprotiline (Ludiomil) |
| Tetracyclic antidepressants                    | maprotiline (Ludiomil)  
mirtazapine (Remeron) |
| Atypical antidepressants                      | nefazodone (Serzone)  
trazodone (Desyrel)  
bupropion (Wellbutrin) |
| Mood stabilizers                              | Lithium (eskalith, lithobid)  
valproate (depakene, deakote)  
carbamazepine (epitol, tegretol)  
lamotrigine (lamictaal) |
| Antipsychotic drugs                           | ziprasidone (Geodon)  
risperidone (Risperdal)  
quetiapine (Seroquel)  
aripiprazole (Abilify)  
asenapine (Saphris)  
paliperidone (Invega)  
amisulpride (Solian) |
| Stimulants                                    | methylphenidate (Ritalin)  
dextroamphetamine (Dexedrine) |
| Dual action antidepressants                   | venlafaxine (Effexor)  
duloxetine (Cymbalta)  
desvenlafaxine (Pristiq) |
1.4.2. **Obesity and resistance to antidepressants**

It is noteworthy that not all patients benefit from antidepressant treatment (Nelson, 1999; Nestler et al., 2002a). Greater relative body weight was found to place patients with major depression at risk for fluoxetine resistance (Prozac) regardless of the severity of depression at baseline (Papakostas et al., 2005). Overweight and obese patients showed a significantly slower response to antidepressant treatment, less improvement in neuroendocrinology and attention and less weight gain than that was showed from patients with normal body weight under antidepressant treatment (Kloiber et al., 2007). This suggests the involvement of unique pathways in depression for the overweight/obese population proposing that new antidepressant drugs targeting these specific pathways are urgently needed. However, further studies in overweight and obese individuals are necessary to further clarify whether the PK of antidepressants is an issue and a reason for the resistance. In this context, obesity was defined as a possible risk factor for the resistance to Prozac treatment in an outpatient study (Papakostas et al., 2005). Given the increasing prevalence of obesity in the general population, studies are needed to better define the role of obesity in depression and specifically on treatment response with standard antidepressants such as SSRIs.

1.5. **The G-protein coupled receptors (GPCRs)**

Most of the receptors targeted by the antidepressants belong to the G-protein coupled receptor superfamily (GPCRs) which is a large and versatile family of cell-surface receptors that participate to the transmission of extracellular signals transduced to the interior of the cell and represents the largest signalling family in the human genome (Lefkowitz, 2004). **Schematic 1.1** represents the main components of the GPCR signalling. GPCRs respond to a remarkable range of stimuli, including neurotransmitters, hormones, and sensory stimuli and they can regulate cell proliferation, differentiation, survival, migration, metabolism, secretory properties, electrical activity, shape, and motility of virtually all mammalian cells and most organ functions. These properties are placing this superfamily of receptors as a master regulator of the human physiology. Phylogenetic analyses show that GPCRs are clustered into five families:
glutamate, rhodopsin, adhesion, Frizzled/Taste2 and secretin (Fredriksson et al., 2003). With more than 1000 unique members (Costanzi et al., 2008) in the human genome, GPCRs represent by far the largest family of cell surface molecules involved in signal transmission, accounting for >2% of the total genes encoded by the human genome. The GPCR family represent the target directly or indirectly of 50-60% of all current therapeutic agents (Rosenbaum et al., 2009; Pierce et al., 2002) as activation, suppression or mimicking the GPCR signaling represents one of the most common themes of currently prescribed therapeutics. Rising evidence suggests that GPCRs may be involved in both the pathogenesis and treatment of mood disorders (Avissar and Schreiber, 2002; Schreiber and Avissar, 2003).

All GPCRs share a characteristic core composed of seven-transmembrane α-helices that weave in and out of the membrane and interact with a wide range of messengers. The N-terminal ends of these receptors are located extracellularly, whereas their C-terminal ends are in the cytoplasm. After ligand binding, GPCRs are activated and expose the intracellular sites for interaction with intracellular molecules. Classically, these receptors stimulate heterotrimeric G proteins, which control the generation of diffusible second messengers and entry of ions at the plasma membrane (Oldham and Hamm, 2008). Each GPCR is coupled to either a different class of G-proteins, Gs, Gi, Gq, or G12/13, or a combination thereof. The binding of the heterotrimeric G proteins to the agonist-occupied receptor catalyses the dissociation of GDP bound to the Gα subunit and its replacement with GTP that leads to the dissociation of the α subunit from the βγ subunits that in turn they signal to several downstream effectors (Pierce et al., 2002). A single GPCR can couple to one or more families of G proteins resulting in the activation of several downstream effectors (Neves et al., 2002). These include classical second messenger pathways such as cyclic adenosine monophosphate (cAMP) controlled by adenylyl cyclases, phospholipases and ionic channels, as well as different kinase cascades (ERK/MAPK, JNK, p38, ERK5) or the Akt/PI3K route. The coupling of a GPCR with a G protein determines the nature and specificity of its downstream signalling targets (Neves et al., 2002). This activation of the receptor also results to the interaction with other cell proteins that drive the initiation of multiple intracellular signalling cascades. Ligands are categorized as full or partial
agonists, that activate the receptor or as antagonists that bound to the receptor and neutrally block the access of the agonist. Despite the vast and longstanding efforts of academic and industrial researchers to pair GPCRs with potential ligands, more than 150 nonsensory GPCRs still remain orphan receptors, for which the cognate ligands have not been identified (Civelli, 2005).

Figure 1.1. The GPCR signalling pathway. Different ligands use G-protein-coupled receptors (GPCRs) to stimulate membrane, cytoplasmic and nuclear targets. GPCRs interact with heterotrimeric G proteins composed of $\alpha$, $\beta$ and $\gamma$ subunits. Typically Gs stimulates adenyl cyclase and increases levels of cyclic AMP (cAMP), whereas Gi inhibits adenyl cyclase and lowers cAMP levels. The members of the $G_\alpha$ family activate phospholipase C (PLC), which cleaves phosphatidylinositol bisphosphate (PIP$_2$) into diacylglycerol and inositol triphosphate (IP$_3$). GABA, gamma-aminobutyric acid; GRK, G protein receptor kinase; P13K, phosphatidylinositol 3-kinase; PKA, protein kinase A; AC, adenyl cyclase; ARB, arrestin; PDE4s, phosphodiesterase4. Figure adapted by (Dorsam and Gutkind, 2007).
1.5.1. The role of arrestins and GRKs in the GPCR signalling

The switch from activation to deactivation of the receptor is not simple and the process of the loss of receptor responsiveness is called desensitization (Lefkowitz, 1993). The agonist binding, in addition to promoting the activation of G proteins and other molecules it also stimulates their phosphorylation by GRKs (G protein-coupled receptor kinases). GRKs is a family of at least 6 GRKs which phosphorylate and regulate a wide variety of receptors that couple to heterotrimeric G proteins (Freedman et al., 1995; Inglese et al., 1993). The physiological relevance and non-redundant role of GRKs has been shown by various gene targeting approaches (Wess, 2000). All GRKs share a central catalytic domain and they can phosphorylate the Ser or Thr residues within the third intracellular loop or cytoplasmic tail of the GPCR which is the region with the G protein interaction. The C-terminal domain of the GRKs is of variable length and seems to facilitate interactions with lipids and other proteins of the membrane.

The membrane recruitment of the GRKs and the phosphorylation of the receptor is a fundamental step for the desensitization process of the GPCRs however it is not sufficient to promote desensitization. Rather, the phosphorylated receptor becomes a substrate for the binding of β-arrestins (Gurevich et al., 1995; Palczewski, 1994). β-arrestins not only mediate the desensitization of GPCRs but they play pivotal role in the internalization of the GPCRs through clathrin-coated vesicles (Laporte et al., 2000) a necessary process for the dephosphorylation, recycling, and resensitization of many GPCRs (Pippig et al., 1995; Krueger et al., 1997; Zhang et al., 1997; Klein et al., 2001; Gaborik and Hunyady, 2004). The evidence for the participation of β-arrestin in GPCR internalization comes from observations that overexpression of β-arrestin can rescue a β2AR sequestration-defective mutant (Ferguson et al., 1996b). The roles of β-arrestin-1 and β-arrestin-2 are very different and distinct between them (Vibhuti et al., 2011). β-arrestins are cytosolic proteins upon binding to GRK phosphorylated GPCRs impair and constrain the communication between the GPCR and its effector G protein. Indeed they block G protein - receptor interaction even in the presence of a GPCR agonist (Ferguson et al., 1996a; Krupnick and Benovic, 1998;
There are two isoforms of β-arrestins, (β-arrestin-1 and β-arrestin-2) that are ubiquitously expressed and each can differentially regulate GPCR desensitization and internalization (Oakley et al., 2000). β-arrestin-1 and β-arrestin-2 are able to both homo- and hetero-dimerize (Milano et al., 2006; Storez et al., 2005).

Although arrestins have long been appreciated to play key roles in the desensitization of function of GPCRs they can also serve as adaptors, scaffolds and/or signal transducers that connect activated receptors with diverse signalling pathways. They can direct the recruitment, activation, and scaffolding of cytoplasmic signalling complexes on the GPCR and evoke signalling pathways different from the action of the G proteins (Shenoy and Lefkowitz, 2005). A much wider range of roles for the β-arrestins, including interactions with receptors outside of the GPCR family and the generation of district, G protein independent signals (DeWire et al., 2007; Gurevich and Gurevich, 2006; Lefkowitz et al., 2006). Proteomic analysis of β-arrestin with their interacting partners revealed more than 200 distinct polypeptides residing in distinct cellular compartments with roles in signal transduction, cellular organization and nucleic acid binding (Xiao et al., 2007) reflecting the complex role of arrestins in the signalling. The essential role of arrestins for the cell signalling and homeostasis has been documented by the phenotypes of β-arrestin null mice (Zamah et al., 2002; Conner et al., 1997; Kohout et al., 2001).

### 1.6. cAMP signalling in depression

3′,5′-cyclic adenosine monophosphate (cAMP) is a ubiquitous second messenger, which is involved in a wide variety of intracellular signal transduction cascades that usually start with a receptor stimulation and play a key role in major biological processes (Beavo and Brunton, 2002; Taylor et al., 2005; Wong and Scott, 2004). The cAMP signalling pathway is subject to regulation by cross-talk through interactions with a number of other distinct signalling cascades at many points that carefully controlled intracellular localization of the signalling components which allows cAMP gradients to be produced in the cell in response to distinct stimuli and environmental conditions (Baillie et al., 2005; Houslay and Adams, 2003; Tasken and Aandahl, 2004; Wong and Scott, 2004; Zaccolo et al.,
2002). The stimulation of cAMP is usually transient and this spatiotemporal control of the cAMP signalling dynamics is achieved by numerous feedback regulations that are activated to finely tune the cAMP signal in intensity, time and its propagation through the intracellular space (Baillie et al., 2005; Houslay and Adams, 2003). cAMP mediated signalling appears to have a key role in the pathophysiology and pharmacology of depression (Perez et al., 2001) as it has been proposed that the mechanism of action for most of the antidepressant treatments involves adaptations at several levels of the cAMP signalling cascade and the upregulation of the cAMP levels (Duman et al., 1997; D’Sa and Duman, 2002). cAMP is generated by adenylyl cyclases (AC) following stimulation of the GPCRs. cAMP exerts its effects through the activation of 3 effectors: protein kinase A (PKA), the exchange protein directly activated by cAMP (EPAC) and the cyclic nucleotide-gated ion channels (Bos, 2006; Dremier et al., 2003) which, in turn, trigger downstream signals that lead to the regulation of various cellular processes including proliferation, differentiation or apoptosis (Tasken and Aandahl, 2004). Chronic electroconvulsive shock and antidepressant drugs have shown to lead to increase coupling of stimulatory G proteins with adenylyl cyclases (Ozawa and Rasenick, 1991), increased levels of cAMP-dependent protein kinases activity (Nestler et al., 1989b; Perez et al., 1989; Perez et al., 1991) as well as increased expression and function of the cAMP response element binding protein (CREB) (Nibuya et al., 1996a).

1.7. Phosphodiesterase gene family

The sole route of cAMP degradation hence termination of its ubiquitous signalling action is achieved via the cyclic-nucleotide hydrolysing phosphodiesterase (PDE) superfamily (Beavo, 1995; Conti and Beavo, 2007; Bender and Beavo, 2006; Maurice et al., 2003). PDEs serve as major regulators of the cAMP signal transduction as they dynamically control its localized pools (Butcher and Sutherland, 1962) to underpin specificity of physiological responses (Houslay et al., 2007). These responses are triggered by cell surface receptors interacting with components of signalling systems or intracellular substrates (Houslay and Baillie, 2005; Houslay and Baillie, 2003). PDEs represent a heterogeneous family of enzymes that are classified into 11 gene families (PDE1-11) (Soderling and Beavo, 2000). Alternative mRNA splicing of these genes generates more than 100
PDEs isoforms in various tissues. The high degree of conservation between these genes implies their important physiological functions. Each PDE subfamily is characterized by a unique sequence homology and primary structure with unique sensitivity to modulators (e.g. \( \text{Ca}^{2+}, \) calmodulin and cGMP) and pharmacological inhibitors (Bender and Beavo, 2006). Different isoforms of PDEs possess different affinities for cAMP and/or cGMP (Stangherlin and Zaccolo, 2012). Three families specifically hydrolyse cAMP (PDE4,7,8), three families specifically hydrolyse cGMP (PDE5,6,9), and the others hydrolyse both cAMP and cGMP (PDE1,2,3,10,11).

They exhibit distinct intracellular distribution with tissue- and cell-specific expression (Nishi et al., 2008) in order to control the cAMP levels in different subcellular compartments and at different times in response to stimuli (Lugnier, 2006; Conti and Beavo, 2007). Within each family, PDEs can have several genes and each gene gives rise to multiple gene products due to alternative mRNA splicing coupled to the use of distinct promoters that all share identical catalytic units.

From all the different cAMP specific phosphodiesterases, PDE4 plays a critical role for the cAMP regulation in the brain (Conti et al., 2003). Elevation of the intracellular levels of cAMP either with rolipram, which is a generic PDE4 inhibitor or stimulation of \( \beta \)-adrenergic receptors, produces an antidepressant effect in animal models (O'Donnell, 1993; O'Donnell et al., 1994; O'Donnell and Frith, 1999; Zhang et al., 2001a). This upregulation of the cAMP has been proposed to lead to a corresponding increase in phosphodiesterase (PDE4) the enzyme that metabolizes the cAMP to AMP and this might be a compensatory mechanism in response to increased cAMP tone (Duman et al., 1997).

### 1.7.1. PDE4 isoforms and structure

Much attention has been focused on the cAMP phosphodiesterase 4 gene family (PDE4) (Conti et al., 2003; Houslay and Adams, 2003). The PDE4 gene family is highly evolutionarily conserved and is encoded by four separate genes namely PDE4A, PDE4B, PDE4C and PDE4D (Dlaboga et al., 2006) all of which have high affinity for rolipram, a PDE4-specific inhibitor (Houslay, 2001). Each one of these
genes gives rise to multiple variants due to alternative splicing (Francis et al., 2001) and the use of multiple different initiation sites resulting in the generation of 25 different isoforms (Houslay et al., 2007; Houslay and Adams, 2003; Johnson et al., 2010). PDE4 isoforms interact with specific proteins/lipids in cells (Houslay and Adams, 2003) and in doing so they play a pivotal role in underpinning the compartmentalization of cAMP signalling (Houslay, 2010; Mongillo et al., 2004). PDE4 is widely distributed but particularly abundant in immune, reproductive and central nervous system. Among the PDEs the PDE4 subfamily represents 70% to 80% of the PDE activity in neuronal tissue (Jin et al., 1999). Due to their localization in the brain, PDE4s are important in the regulation of the intracellular levels of cAMP and are considered to be a prime target for therapeutic intervention for a range of psychiatric disorders such as depression (Cherry and Davis, 1999; Iona et al., 1998). PDE4s are critical components of the cAMP signalling in the brain and responsible in generating microdomains of cAMP leading to spatiotemporal regulation of the cAMP signalling (Houslay, 2010; Oliveira et al., 2010; Manganiello, 2002). PDE4s are the predominant isoforms responsible for the hydrolysis of cAMP formed after b-adrenergic receptor stimulation (Manning et al., 1996) and are involved in the mediation of antidepressant treatment (Ye and O'Donnell, 1996; Ye et al., 1997). Different PDE4 isoforms can associate with agonist bound or unbound GPCRs in various ways; either by direct bind to the receptors (Baillie et al., 2003; Perry et al., 2002; Richter et al., 2008) or with the help of scaffolding proteins such as arrestins that result to the activation of a different signaling cascade (Richter et al., 2008; De Arcangelis et al., 2009; Bouvier et al., 1989).

Each of these four genes encodes multiple proteins that share identical catalytic and C-terminal domains but differ near their N-terminal region. Although much of the sequence of PDE4s is conserved between isoforms, the unique N-terminal region of PDE4 confers direct isoform specific targeting to intracellular signalling complexes (Shakur et al., 1993) and interaction with anchor/scaffold proteins (Conti et al., 2003; Houslay and Adams, 2003; Yarwood et al., 1999; McPhee et al., 1999) allowing the fine-tuning of cAMP signalling to discrete subcellular locations and specific pathways (Bolger, 1994; Conti and Jin, 1999; Houslay, 2001; Houslay et al., 2007). The N-terminal domain of the PDE4s is also responsible for membrane targeting (Shakur et al., 1993; Scotland and Houslay,
1995; Shakur et al., 1995; McPhee et al., 1995). This spatial regulation of the PDE4s appears to underpin the non-redundant functional role of each isoform. This is certainly true when one considers data from approaches that seek to ablate PDE4 activity by siRNA (small interfering RNA) (Lynch et al., 2005; Kolosionek et al., 2009) - mediated knockdown, dominant negative and gene targeting (Houslay, 2010). Gene knock out in vivo studies agree with the non-redundant role of the different PDE4s (Jin et al., 1999; Jin and Conti, 2002; Jin et al., 2005). This is further proven by the identification of the PDE4/anchoring protein/PKA signalling complexes (Bolger et al., 2003a; Dodge et al., 2001; Tasken et al., 2001). Other PDE4s become associated with the GPCRS with adaptor proteins called arrestins (Baillie et al., 2003; Perry et al., 2002; Richter et al., 2008). Except the signalling targeting the N-terminal region, it also exerts a regulatory effect on its catalytic activity (Shakur et al., 1995; McPhee et al., 1995), susceptibility to phosphorylation by PKA (Monaco et al., 1994) and sensitivity to inhibition (Sette and Conti, 1996; Conti et al., 1995; Laliberte et al., 2002; Itoh et al., 2010).

PDE4 isoforms can be subcategorized into dead-short, super-short, short and long as indicated in the Figure 1.2 based upon the presence of regulatory upstream conserved region (UCR) domains located between the isoform specific extreme N-terminal region and the core catalytic domain. PDE4 isoforms can contain up to two upstream conserved regions (UCR1 and UCR2) which are joined together by two linker regions (LR1 and LR2), a highly conserved catalytic unit common to all PDE4s and a C-terminal region, which is unique to each of the four PDE4 subfamilies. The long isoforms possess a full intact UCR and LR regions and the short isoforms have an intact UCR2 but lack UCR1. In contrast, the super-short isoforms lack UCR1 and LR1 but containing a truncated UCR2. The dead-short isoforms do not have the regulatory and linker regions and is both N- and C- terminally truncated. Since it is enzymatically inactive and has survived through evolution, it is speculated that it may be involved in protein scaffolding (Johnston et al., 2004).

Different isoforms of the PDE4 family have been shown to be differentially regulated at the post-translational level. Some forms of PDE4 are regulated by SUMOylation (Li et al., 2010a), ubiquitination (Li et al., 2009a), phosphorylation
(Sette et al., 1994) or via altered expression (Torthy et al., 1992; O'Donnell, 1993; Ye et al., 1997). The electrostatic interaction between UCR1 and UCR2 domains on the long isoforms leads to a regulatory module that is disrupted upon PKA phosphorylation of the UCR1 (Beard et al., 2000). PKA phosphorylation at the UCR1 increases enzymatic activity (Sette and Conti, 1996; Hoffmann et al., 1998; MacKenzie et al., 2002) and sensitivity to rolipram inhibition in vitro (Sette and Conti, 1996; Conti et al., 1995; Laliberte et al., 2002) and in vivo (Itoh et al., 2010) and the phosphorylated and non-phosphorylated form of PDE4 isoforms has been shown to have different binding affinity to rolipram (Fujita et al., 2007). Therefore, phosphorylation of PDE4 by PKA is believed to provide part of the cellular machinery and forms a negative feedback loop whereby localized cAMP levels are further decreased to reduce PKA activity, thereby allowing the dephosphorylation of PDE4 to reset the system thereby leading to cAMP desensitization (Conti et al., 2003).
Figure 1.2. PDE4 isoform diversity: long, short, super-short and dead-short. Each isoform is defined by its unique N-terminal targeting region. Long isoforms have UCR1 and UCR2 domains; short isoforms lack UCR1; super-short isoforms have an N-terminally terminally truncated UCR2; dead-short isoforms lack UCR1 and UCR2 and have an inactive catalytic unit that is both N- and C-terminally truncated. Figure adapted from (Houslay et al., 2007).

1.8. Protein Kinase A as a cAMP signalling effector

The primary effector of cAMP is protein kinase A (PKA) (Skalhegg and Tasken, 2000). PKA mediates a wide variety of cellular functions via direct phosphorylation of a range of substrates at the Ser/Thr amino acids. PKA is a tetrameric enzyme complex, which consists of two regulatory subunits with four encoded genes (Ria, Rib, RIIa, RIIb), and two catalytic (C) subunits with three encoded genes (Ca, Cb, Cg) (Skalhegg and Tasken, 1997). Differential expression patterns of the different PKA subunits can generate a range of holoenzyme combinations each one with different physiochemical properties (Skalhegg and Tasken, 2000). PKA holoenzymes are classified as type I or type II depending on the type of R subunit present (RI or RII) (Scott, 1991). These two PKA types exhibit differential sensitivity to cAMP activation and have different subcellular and tissue distribution. The binding of the cAMP directly to the regulatory
subunits of PKA promotes the dissociation of the active monomeric catalytic subunits which can freely phosphorylate a wide range of protein targets (Taylor and Stafford, 1978). Spatially restricted activation of PKA is conferred to a relevant subset of potential substrates by the binding with AKAPs that they bind to the regulatory subunits of the PKA holoenzyme (Carr et al., 1991; Gold et al., 2006; Kinderman et al., 2006; Huang et al., 1997; Kovanich et al., 2010). AKAPs do not only position PKA inside the cell but they also ensure that this kinase is coupled to its upstream activators, including membrane receptors and ACs, and to signal termination enzymes, such as phosphodiesterases (PDEs) and phosphatases (Coghlan et al., 1995; Dodge et al., 2001; Tasken et al., 2001). The targeting of the two regulatory subunits of PKA by AKAPs is very different suggesting their unique functions (Colledge and Scott, 1999). PKA mediated phosphorylation process represent a highly efficient kinetic amplification mechanism in neurons (Dell'Acqua et al., 2006).

1.8.1. AKAPs as cAMP signalling effectors

Although cAMP is a small molecule that potentially equilibrates throughout the cell in milliseconds the intracellular cAMP concentrations fluctuate transiently within a narrow range upon receptor stimulation (Brooker, 1973). Moreover, several studies have shown that cAMP is not unevenly distributed with dynamic pools throughout the cell (Zaccolo and Pozzan, 2002; Mongillo et al., 2004; Rich et al., 2001; Zhang et al., 2001b; Zhang et al., 2005b; Jurevicius and Fischmeister, 1996). Indeed, imaging studies revealed rise of the cAMP levels selectively in specific cellular compartments upon stimulus in a specific manner that do not diffuse from one compartment to the other, allowing fidelity of the response (Di Benedetto et al., 2008). This diffusion is further prevented either by physical barriers or rapid decay by phosphodiesterases (Houslay, 2010).

The compartmentalization of cAMP signalling is a critical facet of this signalling system and is evident in all cell types. The control of the cAMP flux is governed by the concerted action of two enzyme classes: adenylyl cyclases (synthesize cAMP) and local pools of phosphodiesterases (PDEs) to terminate the signal (hydrolyse cAMP to 5’-AMP). Moreover, this spatial and temporal control of the cAMP signal transduction is further achieved by compartmentalization of
intracellular effectors through adaptors or anchoring proteins (Perino et al., 2012; Scott, 2006; Smith et al., 2006; Scott et al., 2000). A key mechanism by which a common signalling cascade can result in diverse functions is achieved by protein scaffolding complexes. Different ligand binding induces the assembly of distinct macromolecular signalling complexes with transducer scaffold proteins intracellularly of the receptor. Local targeting of an enzyme to a specific subcellular compartment not only ensures that this enzyme is near its relevant targets, but it also segregates its activity by preventing indiscriminate activity to other substrates.

In particular, a family of diverse and well-studied scaffolding proteins are the A-kinase anchoring proteins (AKAPs) that form multi-protein complexes and integrate cAMP signalling with other pathways and signalling events (Beene and Scott, 2007; Murphy and Scott, 1998; Scott, 1991; Scott et al., 2013; Wong and Scott, 2004; Colledge and Scott, 1999; Michel and Scott, 2002).

AKAPs play an important role in the targeting and regulation of PKA-mediated phosphorylation events to specific subcellular locations (Wong and Scott, 2004; Tasken and Aandahl, 2004). In this way, AKAPs help to establish intracellular cAMP gradients, generated via activation of a specific GPCR and uniquely modulated by different subsets of PDEs, resulting in stimulus-specific activation and action of PKA (Di Benedetto et al., 2008; Houslay, 2010; Cooper, 2005; Lefkimmiatis and Zaccolo, 2014; Gold et al., 2013; Bauman et al., 2007). AKAPs consists of a structurally diverse protein family with more than 50 members and with numerous splice variants per gene that are expressed in a cell and tissue specific manner in mammals and lower organism (Pidoux and Tasken, 2010; Wong and Scott, 2004). AKAPs share three common features: first they all contain a PKA-anchoring domain; second they can form scaffold complexes by binding to other signalling enzymes and third they target these scaffold complexes to specific subcellular environments via various targeting motifs, like lipid modifications and protein-protein interaction domains (Wong and Scott, 2004). The first described AKAP that bind to the regulatory subunit of PKA was the microtubule-associated protein MAP2 (Theurkauf and Vallee, 1982). Since
then, many others came in light (Lohmann et al., 1984; Nauert et al., 1997; Carnegie et al., 2004).

In addition to the biological role of AKAPs to bind kinases such as PKA, it has been demonstrated that they can also bind phosphatases (Alto et al., 2002; Lester and Scott, 1997; Edwards and Scott, 2000; Scott, 1997; Coghlan et al., 1995; Schillace and Scott, 1999; Steen et al., 2000). A central mechanism for the regulation of enzymatic activity (Krebs, 1985) and protein interactions (Pawson and Nash, 2000) is achieved by protein phosphorylation regulated by protein kinases and protein phosphatases. This dual-directional process is a versatile means of influencing cellular activity and any breakdown in the signal transduction may lead to pathophysiological outcomes (Cohen, 1999). Therefore, AKAPs can incorporate both kinases and phosphatases in the same signaling complex promoting the anchoring together of both the positive and negative regulators of a common phosphorylation site on the same signalling complex.

Moreover, many AKAPs cluster PKA with PDEs (Dodge et al., 2001; Baillie et al., 2005; Tasken et al., 2001) to tether local cAMP gradients in specific subcellular sites through various PDE binding proteins (Mongillo et al., 2004; Perry et al., 2002). AKAPs bring different phosphodiesterases in different compartments in order to regulate their spatiotemporal action (Tasken et al., 2001; Asirvatham et al., 2004; Willoughby et al., 2006; Yarwood et al., 1999). Scaffold protein like arrestins, even though they do not have the structural or sequence homology with AKAPs, act as AKAPs and interact with phosphodiesterase in order to bring them to specific subcellular compartment (Yarwood et al., 1999; Perry et al., 2002; Verde et al., 2001; Baillie and Houslay, 2005). The importance of co-anchoring both PKA and PDE has been shown via mutants that lack PDE anchoring and this result in sustained PKA activity rather than brief pulses of PKA activity showed under normal conditions (Zhang et al., 2001b). This supports the hypothesis that, in addition to spatially restricting PKA phosphorylation, the complexes composing by AKAP and phosphodiesterases assure that the PKA activity is rapidly quenched by local degradation of cAMP caused by phosphodiesterases.
Thus, spatial and temporal signalling dynamics of cAMP is achieved by various proteins, including PKA, PDEs and facilitated by scaffolding proteins such as A-kinase-anchoring proteins (AKAPs) that provide the structural integrity for multiprotein complexes that often represent hubs for processing of multiple signals (Bauman et al., 2007). These complexes are generated in response to upstream signals that originate from GPCR and adenylyl cyclase activation, creating a complex and continually changing signalling environment in which cAMP levels are distributed unequal in the cell. A further layer of specificity proceeds through protein- or lipid-targeting domains on AKAPs that direct AKAP signaling complexes to intracellular membranes (Skroblin et al., 2010). Therefore, AKAPs complexes contain cAMP-dependent protein kinase, phosphatases, phosphodiesterases and other signalling effector proteins to optimize cellular responses to extracellular signals that cause cAMP or other second messenger alterations (Alto and Scott, 2004; Logue and Scott, 2010). Even though most of the proteins that control the cAMP signal transduction cascade have been identified, there is still the challenge of elucidating the mechanics of their action in real time.

### 1.9. The action of rolipram as an antidepressant

Rolipram, a small-molecule and a generic PDE4 inhibitor is known for its antidepressant action in rodents (Zhang, 2009; Zhang et al., 2006; Przegalinski and Bigajska, 1983; Overstreet et al., 1989; Schwabe et al., 1976; Zeller et al., 1984; Kehr W, 1985; Wachtel, 1982; Wachtel, 1983b; Li et al., 2009b) and in clinical trials (Griebel et al., 1991; Bobon et al., 1988; Wachtel and Schneider, 1986; Wachtel, 1983a; Fleischhacker et al., 1992; O'Donnell, 1993; Bertolino et al., 1988; Hebenstreit et al., 1989). Fig. 1.3 shows the structure of rolipram. Rolipram has also been shown to facilitate the establishment of lasting long term potentiation that leads to cognitive improvement which is another deficit to people with depression (Barad et al., 1998). Chronic administration of rolipram, leads to a sustained elevation of cAMP levels (Schneider, 1984) and increases the expression of CREB, BDNF and TrkB, all of which are believed to facilitate the action of antidepressants (Li et al., 2009b; Nibuya et al., 1996a). Rolipram induced elevation of intracellular cAMP levels is responsible for the increased
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synthesis and release of norepinephrine which enhance central noradrenergic transmission (Schwabe et al., 1976; Wachtel, 1983b). Rolipram is believed to increase the neurotransmission of noradrenaline in two ways: 1. presynaptically by increasing the synthesis and release of norepinephrine (Kehr W, 1985) and 2. postsynaptically by the increase availability of cAMP via the inhibition of PDE4s that modulate the action of hormones, neurotransmitters and drugs (Krebs and Beavo, 1979; Wachtel and Schneider, 1986). As rolipram exerts its effects both pre- and post-synaptically, it induces rapid anti-depressant effects compared to other antidepressants (Wachtel, 1983b). Co-administration or rolipram with other antidepressant has been shown to shorten the time required for cAMP response element-binding protein (CREB) upregulation and BDNF expression both indicators for antidepressant action (Nibuya et al., 1996a). Enhancement of the cAMP response due to rolipram could be particularly advantageous if the serotonin and noradrenergic receptors coupled to cAMP are downregulated in response to elevated levels of monoamines, as reported for the beta adrenergic receptors (Duman, Heninger et al. 1994) and the 5-HT7 receptor (Sleight, Carolo et al. 1995). In addition, rolipram has been shown to be an ideal drug candidate for brain disorders as PET imaging revealed the ease with which it crosses the blood brain barrier (BBB) (Fujita et al., 2005). This confers a competitive advantageous characteristic over other comparable antidepressants.

Figure 1.3. Structure of rolipram. Image shows the chemical structure of the phosphodiesterase inhibitor rolipram.

However, despite initial promise, the therapeutic potential of rolipram as an antidepressant is limited due to the side effects of nausea and emesis (Scott et al., 1991; Zeller et al., 1984; Rock et al., 2009; Robichaud et al., 2001) owing to its mechanism of action as a non-isoform-specific PDE4 inhibitor (Dyke and Montana, 2002; Robichaud et al., 2002; Hirose et al., 2007; Bertolino et al., 1988; Hebenstreit et al., 1989). The molecular identification of the isoform that mediates the antidepressant action of rolipram and the creation of selective
inhibitors of PDE4 isoforms may have therapeutic effects with minimal adverse reactions (O'Donnell and Zhang, 2004). Structural analysis of the catalytic domains of PDE4A, PDE4B, PPDE4C and PDE4D bound to inhibitors revealed significant conformational differences between the different enzymes that can shed light for the development of selective inhibitors for the different members of the PDE4 family (Wang et al., 2007). Despite the fact that the design of specific PDE4 inhibitors has been very slow, it has already been achieved for the sub-family of PDE4Ds in order to enhance cognition (Burgin et al., 2010; Houslay and Adams, 2010). To date, no PDE4 inhibitor has been brought to market as an antidepressant drug due to issues related to tolerability and side effects (Giembycz, 2005).

1.10. Brain regions involved in depression

The neurocircuitry of depression is complex and involves portions of the limbic system such as hippocampus, amygdala, thalamus, cortex and other structures of the emotional brain to play a role in the pathophysiology of depression (Price and Drevets, 2010; Eisch and Petrik, 2012). Increasingly sophisticated methods of brain imaging such as positron emission tomography (PET), single photon emission computed tomography (SPECT) and functional magnetic resonance imaging (fMRI) have allowed the imaging of the brain in vivo while it functions over tasks. An fMRI scan tracks the changes in a region of the brain in response to various tasks. A PET or SPECT scan maps the brain by measuring the distribution and density of neurotransmitter receptors in brain regions. Using these powerful techniques studies found that depressed patients exhibit structural and functional changes in brain regions important for learning and memory such as prefrontal cortex (Dolan et al., 1993; Drevets, 2000), hippocampus and amygdala (Sheline et al., 1998; Videbech and Ravnkilde, 2004).

Most of the studies are focusing on hippocampus that is significantly altered in depression and known to regulate mood and cognition (Pittenger and Duman, 2008; Sapolsky, 2003). Hippocampus have received much attention in the recent years as human imaging and rodent studies showed that depression is associated with decreased hippocampal volume and atrophy of neurons (Pittenger and Duman, 2008; Fossati et al., 2004; Bremner et al., 2000; Sheline et al., 1996;
Duman and Aghajanian, 2012). The reduction of the hippocampal volume observed in patients with depression has been speculated to cause the memory impairment in these patients (Czeh and Lucassen, 2007). Interestingly, experimental obesity on the brain in mice revealed decreased myelin levels and marked alterations to the fatty acid composition of myelin in genetically obese mice (Sena et al., 1985) and regression studies associate increased body mass index with decreased brain volume (Ward et al., 2005) suggesting another link between obesity and depression.

Amygdala, a structure of the limbic system deep in the brain that’s associated with emotions such as anger, pleasure and fear (Fernando et al., 2013). Amygdala has been shown to be activated when a person recalls emotionally charged memories, such as frightening situation (Duvarci and Pare, 2014). Clinical studies revealed that the activity in amygdala is higher when a person is either sad or clinically depressed. Interestingly, this increased activity continues even after recovery from depression (Foland-Ross and Gotlib, 2012). The prefrontal cortex (PFC) is implicated in executive tasks and reward and recently it has been proposed to be involved in the control of emotions and the pathophysiology of depression (Krishnan and Nestler, 2008; Nestler and Carlezon, 2006).

However even though isolated brain regions have been studied in relation to depression (Eisch and Petrik, 2012) the connectome and how they are interconnected between each other and the other the brain regions involved in depression and where do they send their signals is totally unknown.

1.10.1. The role of hypothalamus in mood disorders

Despite a brain preference for glucose as its primary energy source, more recent evidence suggests that hypothalamic lipid metabolism and signalling have significant roles in regulating energy balance, food intake and the aetiology of metabolic diseases (Lam et al., 2005b; Lopez et al., 2005). The central nervous system has developed mechanisms in order to sense nutrient availability, regulate and connect main physiological homeostatic responses peripherally and centrally (Lam et al., 2005b). The hypothalamus, the main regulator of energy
homeostasis is a brain region highly vascularized with a lot of communication with the periphery that has been involved in both obesity and depression. Many hormones or macronutrients in the circulation can bind to specific receptors in the hypothalamus in order to regulate different signalling pathways. For instance, it has been shown that leptin, a main adipose tissue hormone, can be transported across the BBB through a substrate concentration dependent manner and signal in the hypothalamus feeding circuits that regulate appetite (Bouret, 2009).

Considering progress has been made to elucidate the key role of hypothalamus in regulating the cellular and molecular processes comprising the circuits regulating energy homeostasis (Ryan et al., 2012; Schwartz et al., 2000; Woods and D’Alessio, 2008). This homeostatic regulation integrates acute satiation signals, arising in the gut and secreted phasically during meals, with more tonically active adiposity signals to appropriately adjust nutrient intake and energy expenditure (Ryan et al., 2012; Schwartz et al., 2000; Woods and D’Alessio, 2008). Due to this, many studies have investigated signalling pathways in the hypothalamus that are critical in nutrient sensing. Also those that regulate the complex network of signals impacting on both energy metabolism and feeding behaviour, in order to influence energy homeostasis both centrally and peripherally (Cota et al., 2007; Le Foll et al., 2009; Moran, 2010; Lam et al., 2005b; Woods et al., 1998; Schwartz et al., 2000; Ahima et al., 1996; Wang et al., 1998; Obici et al., 2002). The quality of dietary FA during postnatal life affects permanently the development of the central regulatory circuits in the hypothalamus that control energy balance and may do so through a leptin-independent mechanism (Schipper et al., 2013).

The specific role of different fatty acids in the hypothalamic area for the regulation of energy and appetite has been studied as well (Levin et al., 1999; Jo et al., 2009; Huang et al., 2004; Wang et al., 2002; Morgan et al., 2004). Interestingly, it has been found that dietary fatty acids differentially affect signalling pathways in the hypothalamus and modulate overall energy expenditure as polyunsaturated fatty acids (PUFAs) have been shown to increase pro-opiomelanocortin (POMC) expression and decrease neuropeptide Y (NPY) expression, both peptides with important roles in appetite, compared to diets
higher in saturated fats (SFAs) (Dziedzic et al., 2007; Huang et al., 2004) suggesting a differential role for the different degrees of saturation of fatty acids.

### 1.11. Mechanisms involved in the action of antidepressants

Extensive biochemical research in mood disorders in order to elucidate the action of antidepressants has focused along the cascade of events involved in signal transduction, from the level of the monoamine neurotransmitter to the level of the neurotransmitter receptors and lately to information transduction mechanism beyond receptors involving the coupling of receptors with signal transducers. However, the precise mechanism of action of antidepressant drugs remains unclear. Several mechanisms have been proposed to explain the action of antidepressants with the main focus on the increase of the serotonin and norepinephrine in the brain as alterations of these neurotransmitter receptors appears to play a major role in the pathogenesis of depression (Mann, 1999; Ressler and Nemeroff, 1999). A possible explanation for the action of different antidepressant treatments is that they affect a common intracellular signal transduction pathway beyond the levels of serotonin and norepinephrine and their relative receptors, possibly through cross-talk between different neurotransmitter/receptor systems. If that’s the case, the regulation of the monoamines and their receptors represent an initial effect that subsequently leads to the regulation of postreceptor signalling pathways that mediate the therapeutic action of antidepressants (Sulser, 1989; Hudson et al., 1993; Duman et al., 1994).

A major candidate for the post-receptor target that could be regulated by both serotonin and norepinephrine and thereby mediate the long term adaptations in response to diverse antidepressant treatments is the nuclear transcription factor cAMP response element binding protein (CREB). CREB mRNA and protein phosphorylation is upregulated after chronic but not acute antidepressant treatment in the hippocampus (Nibuya et al., 1996b; Thome et al., 2000; Tiraboschi et al., 2004) suggesting a chronic effect of the treatment on CREB. Chronic agonist exposure due to antidepressant treatment causes desensitization
of the receptors which can be manifested clinically as tachyphylaxis often involving a decrease in the net number of post synaptic receptors at the cell surface including β-adrenergic and serotonin receptors (Sulser et al., 1984; Katz, 2011). This may involve β-arrestins or may be mediated through other distinct processes. However, even though this reduction has been documented it is possible that there is an increase in the functional output of these receptor coupled intracellular pathways. Therefore, even though the density and function of these receptors is reduced, they are not eliminated by chronic antidepressant treatment suggesting that the activation of the remaining receptors is sufficient enough to stimulate the intracellular signal transduction pathways (Nibuya et al., 1996a). The decreased density in monoamine receptors upon antidepressant treatment is also observed in cell culture systems that lack presynaptic input (Fishman and Finberg, 1987; Kientsch et al., 2001). Another possible explanation for reduction of functional monoamine receptors could represent a compensatory response to the high levels of the different neurotransmitters in the synapse.

Depression has been described as a neurodegenerative disease as there is a commonly observed delay of several weeks between the start of the antidepressant treatment (Belmaker and Agam, 2008) or ECT to exert their therapeutic response and this might reflect the requirement for serotonergic or noradrenergic nerve-terminal regrowth (Mamounas et al., 1995). This hypothesis is in agreement with observation that different treatments in order to exhibit a beneficial effect and ameliorate the symptoms of depression they need to act over the course of weeks or months (Baldessarini, 1989; Wong and Licinio, 2001). This delay of their therapeutic effects has led to many studies investigating the long term pharmacology effects of antidepressants (Mongeau et al., 1997). Chronic antidepressant treatment is accompanied by transcriptional and cellular modulation, occurring on a later timescale than the changes in monoamine levels which is consistent with the onset of the relief of symptoms (Frazer and Benmansour, 2002; Nestler et al., 2002a; Young et al., 2002; Carlezon et al., 2005; Blendy, 2006). However, the acute effects of antidepressants in the synapse although not sufficient by themselves, initiate the cascade of events that result in clinical improvements (Miller et al., 1996). Furthermore, the
generation of fast-acting and more efficacious class of drugs may be proven beneficial for the treatment of severe or depression associated with suicide.

Moreover, antidepressant drugs possess no mood elevation or euphoric feelings in healthy individuals but correct the mood disturbances in depressed patients (Nelson, 1999) indicating that the action of antidepressant drugs that underlie their clinical results uncovers only under pathophysiological conditions sharing similar aspect of depression.

1.12. CREB as an intracellular target for the regulation of depression

CREB is a member of the basic region/leucine zipper motif (Bzip) superfamily of transcription factors that is being activated by phosphorylation at Ser133 and in turn influences the transcription of genes that encode a wide range of functions. Although the particular CREB-regulated genes that contribute to depression have not been identified there are some plausible targets such as DARPP32 (Cantrup et al., 2012; Helms et al., 2006) and BDNF (Pittenger and Duman, 2008). CREB activation is increased by the cAMP cascade due to its phosphorylation by the cAMP effector, PKA (Nibuya et al., 1996a) and this leads to the transcriptional regulation of a large cohort of genes. However, there is huge controversy whether phosphorylation of CREB has an anti- or pro- depressive effects (Carlezon et al., 2005). Chronic antidepressant treatment increases the phosphorylation of CREB in certain brain regions (Thome et al., 2000; Tiraboschi et al., 2004; Nibuya et al., 1996a) as well as genes regulated by CREB such as BDNF (Nibuya et al., 1995). Virus mediated overexpression of CREB in the hippocampus produced antidepressant behaviour in acute behavioural tests (Chen et al., 2001). Both these observations suggest that CREB mediates some of the gene transcription changes that are being induced by chronic antidepressant treatment. However, activation of CREB has also been shown to play an opposing role. Chronic antidepressant treatment has been shown to decrease CREB phosphorylation in certain brain regions (Kuipers et al., 2006; Manier et al., 2002) as well as the expression of CRE-containing genes such as tyrosine hydroxylase (Nestler et al., 1990), corticotropin-releasing hormone (Brady et al., 1991) and β-adrenoreceptor (Banerjee et al., 1977; Meyerson et al., 1980). Virus
mediated overexpression of CREB in the nucleus accumbens has been shown to increase immobility in the forced swim test an indicator for depression phenotype (Pliakas et al., 2001). Stress also increases CREB activity and the likelihood of depression in transgenic CRE-luciferase reporter mice (Boer et al., 2007a). Lastly, CRE-deficient (CREBADmutant) mice demonstrate increased hippocampal neurogenesis and an antidepressant phenotype in the tail suspension and forced swim test (Gur et al., 2007). Therefore, whether activation of CREB has an anti or pro depressant effect is still unclear.

Much experimental work has focused on the phosphorylation of CREB on either the serine-199 of CREB-327 isoform or serine-133 of CREB-341 isoform in the brain following anti-depressant treatment (Kuipers et al., 2006; Tardito et al., 2006; Thome et al., 2000; Tiraboschi et al., 2004). While this phosphorylation event promotes the recruitment of CREB coactivator CBP (Shaywitz and Greenberg, 1999) and is required for CREB activation, it is not sufficient for CREB transcriptional activity (Boer et al., 2007b; Ravnskjaer et al., 2007; Schwaninger et al., 1993). Hence a better understanding of the posttranslational regulation of CREB in depression might advance our knowledge on its function in depression-related brain signalling.

1.13. Serotonin and noradrenergic molecular pathways in depression

It is speculated that antidepressant drugs regulate CREB either via the activation of the cAMP cascade (via 5-HT\textsubscript{4,6,7} and α1 adrenergic receptor) or by Ca\textsuperscript{2+} activated protein kinases (5-HT\textsubscript{2} and α1 adrenergic receptors) (Shelton, 2007). The serotonin pathway is a key modulatory pathway in a plethora of functions of the central nervous system in physiological and disease states (Gingrich and Hen, 2001; Barnes and Sharp, 1999). The plasma tryptophan is the precursor for the synthesis of serotonin in the brain, and it is transported into the brain across the blood brain barrier (BBB) (Blomstrand, 2001). Numerous reports implicate the serotonin pathway in controlling the neuronal functions and to be responsible for the cognition and behaviour (Struder and Weicker, 2001). Serotonin receptors are involved in several signalling intracellular transduction pathways one of them being the regulation of the cAMP pathway (Raymond et al., 2001) that has
been suggested to play a role in depression. The hypofunction of the serotonergic system is thought to play a major role in the development of depression (Middlemiss et al., 2002) and many other neuropsychiatric disorders (Barnes and Sharp, 1999; Gingrich and Hen, 2001; Duman et al., 1997; Manji et al., 2001) that is treated predominantly with SSRIs or MAOIs. SSRIs, except their role in increasing the amount of serotonin in the synapse it has been speculated that they also act via the desensitization of the serotonin autoreceptor. Due to the recognition of the role of the serotonin in depression different KO mice were generated in the serotonin pathway in order to study the role of the different molecules in the serotonin pathway such as serotonin transporter and various serotonin receptors (Gardier, 2009).

The pathophysiology of the adrenoreceptor pathway has been involved in depression. There is an inverse correlation between the severity of depression and B-adrenergic receptor density (Jeanningros et al., 1991) and a negative association between B-adrenergic receptor stimulated cAMP activity and depressive symptoms (Mazzola-Pomietto et al., 1994). Down-regulation or desensitization of B-adrenoreceptor is dependent on the phosphorylation mediated by cAMP dependent protein kinase A (PKA) and B-adrenergic receptor kinase (GRK). Antidepressants have been shown to modulate the density of the functional neurotransmitter receptors such as B-adrenergic receptors in the brain (Banerjee et al., 1977; Peroutka and Snyder, 1980) as well as in cultured cells (Honegger et al., 1986; Fishman and Finberg, 1987). B-adrenergic receptor down regulation is accompanied by decreased receptor-stimulated cAMP formation (Vetulani and Sulser, 1975) however the mechanism for this reduction is not understood. On the contrary it has also been shown that high densities of brain α2 adrenoreceptor in the brain of depressed suicides and many antidepressants are able to modulate the density and/or function of these inhibitory receptors (Gonzalez-Maeso et al., 2002).

Dopaminergic signalling has been involved in the pathophysiology of depression (Dunlop and Nemeroff, 2007). Acute antidepressant treatments have no consistent effect on dopaminergic function however increased dopaminergic function has been observed following chronic antidepressant treatments (Willner, 1983). The site of these effects is at present unclear, but appears to be
beyond the dopaminergic receptors hypothesizing that these effects happen via the decreased sensitivity of pre-synaptic dopaminergic autoreceptors (Willner, 1983). Figure 1.4 shows the two main pathways involved in depression and their downstream effectors.

**Figure 1.4.** Schematic of the main pathways involved in depression. Schematic of the main pathways that are involved in depression. The schematic represents the major components of the β-adrenergic and serotonin system both of which lead to the activation of PKA that phosphorylates CREB and this results in the translocation of CREB in the nuclei in order to regulate the transcription of different genes.

### 1.14. The role of p11 in depression

p11, also called S100A10, is a member of the S100 EF-hand protein family (Donato, 1999) and a small acidic protein that has been involved in depression due to its interaction with specific serotonin receptors as it increases their cell surface localization and regulate their trafficking and signalling (Svenningsson et
al., 2006; Warner-Schmidt et al., 2010; Warner-Schmidt et al., 2009). p11 is a member of the S100 family of proteins, and a key regulator of depression and antidepressant response to rodents (Svenningsson et al., 2006; Warner-Schmidt et al., 2010; Warner-Schmidt et al., 2009) as p11 mRNA and protein levels are decreased in brain tissue of depressed patients (Svenningsson et al., 2006; Anisman et al., 2008) and in animal models of depression (Svenningsson et al., 2006). Antidepressant or electroconvulsive therapy increases the levels p11 (Svenningsson et al., 2006). Overexpression of p11 in a transgenic mouse model recapitulates certain behaviours seen after antidepressant treatment in mice (Svenningsson et al., 2006). p11 knock out mice showed a depression phenotype with reduced responsiveness to serotonin receptor agonists as well as resistance to behavioural effects upon antidepressant treatment (Svenningsson et al., 2006). Therefore a modulation of the serotonin receptor by p11 might be involved in molecular adaptations occurring in neuronal networks that dysfunction in depression. Interestingly, cytokines can increase the p11 levels which in turn leads to an antidepressant effect in mice (Warner-Schmidt et al., 2011) implying that the brain cytokines might also exert an antidepressant action. Non-steroidal anti-inflammatory drugs (NSAIDs) can reduce the levels of cytokine secretion (Bacchi et al., 2012). This is in accordance with the fact that widely prescribed NSAIDs antagonize both biochemical and behavioural responses to SSRIs (Warner-Schmidt et al., 2011) as increased levels of different cytokines induced by antidepressant treatment can be inhibited by anti-inflammatory agents and this reverses the antidepressant effect (Warner-Schmidt et al., 2011).

1.15. The role of neurotrophins in depression

Recently, deficiencies in neurotrophin production have been implicated in the pathophysiology of depression and has been suggested that antidepressant treatment and ECT might work by boosting their production in the brain. This hypothesis suggests a ‘neurotrophinboosting’ pharmacotherapy for depression that requires nerve growth for a clinical response.

The role of neurotrophins, a family of growth factor hormones has been extensively studied in the regulation, development and maintenance of the
peripheral (PNS) and central (CNS) nervous system. Chronic infusions of neurotrophins such as brain derived neurotrophin receptor (BDNF) or neurotrophin 3 (NT-3) into the rat midbrain increased the turnover rate of serotonin and noradrenaline in many brain regions such as neocortex, basal ganglia and hippocampus (Martin-Iverson et al., 1994; Altar et al., 1994; Siuciak et al., 1996). This in turn, promoted the function, sprouting and regrowth of 5-HT-containing neurons (Mamounas et al., 1995). This effect of BDNF and NT-3 on serotonergic neuronal function, growth and regeneration suggested a potential link between neurotrophins and depression as the serotonergic system is altered in people with depression. This link was also based on the observations that the brain tissue as well as the ventricular fluids of patients with major depression have reduced serotonin turnover (Asberg et al., 1976; Mann et al., 1989; Risch and Nemeroff, 1992).

One of the neurotrophins that its expression and/or function has been shown to be involved in the pathophysiology and pharmacotherapy of depression is the BDNF (Pittenger and Duman, 2008; Tardito et al., 2006; Kozisek et al., 2008; Chen et al., 2006; Sakata et al., 2010) as well as its receptor tropomyosin-related kinase B (TrkB). TrkB expression is regulated by PKA activation and CREB binding (Deogracias et al., 2004). BDNF is a peptide critical for axonal growth, neuronal survival, and synaptic plasticity and function.

BDNF levels have been reported to be reduced in patients with depression, and antidepressants as well as physical exercise seem to upregulate BDNF (Karege et al., 2005; Dias et al., 2003; Tsankova et al., 2006; Russo-Neustadt et al., 2004). Chronic rolipram co-administration with antidepressant has also been shown to induce BDNF upregulation in the hippocampus (Fujimaki et al., 2000). It has been reported that BDNF administration is sufficient to produce an antidepressant effect (Nibuya et al., 1996a; Adachi et al., 2008). Peripheral BDNF administration induces an antidepressant effect in mice accompanied by increased neurogenesis, increased hippocampal BDNF and p-CREB (Schmidt and Duman, 2010). Infusions of BDNF into the brain can accelerate the regrowth of serotonergic nerve fibres following their destruction by a serotonergic neurotoxin (Mamounas et al., 1995). Therefore, positive modulators of BDNF/TrkB signalling might result to a potential antidepressant effect in humans.
BDNF signalling in the brain is thought to have a potential antidepressant action by involving the hippocampus and HPA axis (Castren and Rantamaki, 2010; Wang et al., 2008). BDNF has also been involved in obesity as dendritically targeted BDNF transcript in the hypothalamus is important for the regulation of food intake in mice (Liao et al., 2012).

However, conflicting findings have been reported for BDNF expression level depending on the length of the treatment as well as the time interval following administration. Short-term antidepressant treatment decreased BDNF expression in rodents whereas long-term increased BDNF levels (Coppell et al., 2003; De Foubert et al., 2004; Khundakar and Zetterstrom, 2006). This biphasic induction of BDNF of the different transcripts of BDNF was also shown in vitro using a human hippocampal cell line suggesting a common feature between rodents and humans (Donnici et al., 2008).

The role of TrkB, the receptor of BDNF in depression is not clear. Preventing BDNF production in dopaminergic regions or inhibiting BDNF/TrkB signalling in the nucleus accumbens induces an antidepressant effect (Eisch et al., 2003). Activation of TrkB has a depressant effect as administration of a low molecular weight ligand for TrkB (ANA-12) that acts as an antagonist (prevents the activation of the receptor) for the TrkB demonstrated an antidepressant effect in mice (Cazorla et al., 2011). Therefore, a better characterization of the time and dose of BDNF and its role in depression in needed.

1.16. Neurogenesis as a potent antidepressant mechanism

In mammals, cell proliferation and neurogenesis in the hippocampus happens throughout the adult mammalian life. A possible mechanism of action of antidepressants is via anti-apoptotic and neurotrophic effect of antidepressants as people with depression exhibit neuronal loss (Drzyzga et al., 2009). More specifically, cell loss, atrophy and decreased neurogenesis in the hippocampus has been implicated in the pathophysiology of depression and a direct role of the adult neurogenesis in depression has been described (Snyder et al., 2011; Eisch and Petrik, 2012). Studies in animal models of depression suggest that reduced
neurogenesis in the adult hippocampus might contribute to structural changes and to alterations of the behaviour of these animals. Increased expression of CREB and induction of BDNF and TrkB in response to chronic antidepressant treatments could enhance the survival and/or function of neurons as it has been reported for direct application of BDNF (Ghosh et al., 1994; Lindsay et al., 1994; Lindvall et al., 1994). Hippocampal neurogenesis can be driven by the administration of antidepressants (Malberg et al., 2000; Santarelli et al., 2003; Sairanen et al., 2005) or electroconvulsive seizure (Madsen et al., 2000) suggesting that correlation of increased neurogenesis with antidepressant treatment. Increased hippocampal neurogenesis has been used as an indicator for antidepressant behaviour (Newton et al., 2002).

However, behavioural studies suggest that antidepressant treatment exert their therapeutic effects via both neurogenesis-dependent and -independent pathways (Santarelli et al., 2003; Sahay and Hen, 2007; David et al., 2009; Holick et al., 2008).

Among the various molecules assigned to contribute to adult neurogenesis and cognition, the most important is cAMP response element-binding protein (CREB) (Mizuno et al., 2002) CREB is a transcription factor that rapidly responds to intracellular signalling effectors originating from neurotransmitters and neuronal growth signalling in mature neurons (Lonze and Ginty, 2002). BDNF is regulated transcriptionally by CREB and except its role in learning and memory it also promotes cell proliferation as intra-hippocampal infusion of BDNF promotes adult neurogenesis (Scharfman et al., 2005).

Rolipram, a generic PDE4 inhibitor increases the cAMP and phosphorylation of CREB and rolipram’s antidepressant effect depends partially on its neurogenic action in the hippocampus (Li et al., 2009b; Schneider, 1984). Chronic, but not acute, treatment with rolipram increases hippocampal proliferation and survival of newborn neurons in the dentate gyrus and this is mediated by cAMP/CREB (Nakagawa et al., 2002a; Nakagawa et al., 2002b; Fujioka et al., 2004; Sasaki et al., 2007); this effect is accompanied by the activation of CREB (Fujioka et al., 2004; Nibuya et al., 1996b; Nakagawa et al., 2002b; Zhu et al., 2004). Rolipram is a generic inhibitor for PDE4 isoforms for all four sub-families (A/B/C/D).
Indeed targeted gene knock down of \textit{PDE4D} in mice generates animals that are characterized by an increase neurogenesis and phosphorylation of CREB (Li et al., 2011). Rolipram has also been associated with memory enhancing effects in rodents (Barad et al., 1998; Zhang and O'Donnell, 2000; Zhang et al., 2000; Zhang et al., 2004; Zhang et al., 2005a), which are associated with the increased phosphorylation of CREB in the hippocampus (Blendy, 2006; Monti et al., 2006; Sairanen et al., 2007).

1.17. New strategies and targets for the development of antidepressant treatments

The commonly used antidepressant medicine treatments have been developed 50 years ago targeting the monoamines. Unfortunately \textasciitilde50\% of individuals with depression show full remission in response to these mechanisms. Therefore new strategies for the development of antidepressant treatment that do not focus on monoamines are urgently needed (Berton and Nestler, 2006). The mechanism of action underlying the targets beyond monoamines signalling is now emerging in both preclinical and clinical reports in depression (Wong and Licinio, 2004; Hunsberger et al., 2007).

Many of these new target molecules have been found in the hypothalamus underlying the important role of this brain region in depression. However the depression related hypothalamic nuclei or neuronal populations inside the hypothalamus critical for the depression phenotype remains to be determined. The hypothalamus is a brain region that receives most of the sensory information and directs it to the appropriate part of cerebral cortex that regulates higher functions such as speech, behaviour and movement. Bipolar disorder has been suggested to result from problems in the thalamic area that helps link sensory input to pleasant and unpleasant feelings.

More genes in the paraventricular nucleus (PVN) of hypothalamus are starting to be recognized in depression such as specific alleles of the galanin which is a neuropeptide found in the hypothalamus that has been proposed to link obesity and depression (Davidson et al., 2011) and has been shown to interact with the serotonin receptors (Kuteeva et al., 2010).
Melanin-concentrating hormone (MCH) is a 17 amino acid peptide hormone that is found in the cell bodies in the nucleus accumbens of hypothalamus that acts via the binding to the MCHR receptors. It was found that acute and sub-chronic administration of a selective antagonist for MCHR1 showed an antidepressant effect in rats and mice (Gehlert et al., 2009).

Another molecule that has been shown to play a role in the pathophysiology of depression is the adipocyte derived hormone adiponectin (Liu et al., 2012) that acts by binding to its receptor in the hypothalamus (Kubota et al., 2007). It has already been described that the plasma levels of adiponectin are reduced in major depression (Leo et al., 2006). Plasma levels of adiponectin are also reduced in a mouse model of depression. Administration of adiponectin either on normal weight as well as diet induced obese mice produces an antidepressant effect suggesting a critical role of adiponectin in depression and point out a potential innovative therapeutic approach for depressive disorders (Liu et al., 2012).

Exercise has been shown to have an antidepressant effect on depressed patients (Strawbridge et al., 2002) as well as in animal models of depression (Bjornebekk et al., 2006). It is believed that exercise exerts its antidepressant effect by upregulating neurotrophic factors as well as inducing cell proliferation and neurogenesis in hippocampus (Bjornebekk et al., 2005). Studies with enriched environments also induce an antidepressant effect in mice (Hattori et al., 2007) and it is believed to happen by increasing the number of new neurons in the adult dentate gurus (Kempermann et al., 1997).

Vascular endothelial growth factor (VEGF) signalling has been shown to be required for the behavioural actions of antidepressant treatment of monoamine reuptake inhibitors in the hippocampus (Greene et al., 2009a). The hippocampus has attracted a lot of attention due to its role in depression and its central function of the limbic system. Neuritin, an activity dependent gene that regulates neuronal plasticity in the hippocampus, is decreased with chronic stress and is upregulated after antidepressant treatment (Son et al., 2012). Moreover, neuritin overexpression produces an antidepressant effect whereas
neuritin knock down produces a depression like behaviour (Son et al., 2012). Neuritin has also been involved in the final output of the action of BDNF (Naeve et al., 1997; Wibrand et al., 2006).

Recently, it has been described that depressed patients have increased expression of the mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) which is a negative regulator for the MAP kinase (Duric et al., 2010) suggesting potential targeting of the MAPK signalling might recapitulate an antidepressant effect.

1.18. Western but not Mediterranean diet correlates with depression

Despite their high caloric contents, western diets are poor in essential nutrients for the brain such as ω-3 PUFAs and notorious for their high levels of ω-6 PUFAs, saturated fatty acids, trans fat and sugar contents that result in malnutrition. Such dietary customs critically contribute to the high prevalence of chronic diseases such as obesity and metabolic disorders that plagues westernized countries. Adherence to an overall Mediterranean diet has been suggested to play a protective role for depression phenotype. A beneficial role of unsaturated fatty acids such as PUFAs (Mozaffarian et al., 2010) and olive oil (Fernandez-Jarne et al., 2002; Kontogianni et al., 2007; Barzi et al., 2003) found in the Mediterranean diet concerning depression as they are considered healthy lipids. Numerous studies supporting the positive relationship between improved nutrition and better mental health (Freeman, 2010; Appleton et al., 2010; Skarupski et al., 2010; Gilbody et al., 2007). It is believed PUFAs increase the fluidity of the neuronal membrane and this gives easier access of the neurotransmitters to their receptors fluidity (Horrocks and Farooqui, 2004). ω-3 fatty acids are suggested also to be inhibitors for the production of cytokines (Endres et al., 1989; Oh et al., 2010; Robinson et al., 1996). Figure 1.5 shows the main categories of fatty acids. Fatty acids are divided in two main groups: the saturated (SFAs) and unsaturated fatty acids (UFAs). The unsaturated fatty acids can be further divided in two main categories the monounsaturated (MUFAs) and the polyunsaturated (PUFAs) fatty acids. The PUFAs are further
classified into ω-3 (n-3), ω-6 (n-6) and ω-9 (n-9) groups. The parent essential fatty acid of ω-3 PUFA is α-linoleic acid (ALA; C18:3n-3), and that of the ω-6 group is linoleic acid (LA; C18:2n-6).

**Figure 1.5. Schematic of the different fatty acid categories.** The two main categories for the fatty acids are the saturated and unsaturated fatty acids. Moreover, the unsaturated fatty acids are divided into monounsaturated and polyunsaturated fatty acids. The unsaturated fatty acids are divided into monounsaturated and polyunsaturated fatty acids.

Recent studies have revealed a positive correlation between western diet consumption and depression. Studies in humans are in accordance with studies in animals, showing that high fat diet consumption was accompanied with a depression phenotype and altered serum lipids (Chilton et al., 2011). Interestingly, at the same study it was observed a negative correlation between BMI and depression as well as only a subgroup of monkeys developed a depression phenotype, which implies that a high fat western diet is likely to
increase depressive behaviour among subgroups of susceptible individuals rather than universally (Chilton et al., 2011). Moreover, HFD exacerbated the depressive like behaviour in the FSL (Flinders sensitive line) rats (an inbred genetic model of depression) but conversely tended to reduce anxiety levels and impair cognition in the FSL rats (Abildgaard et al., 2011). The behavioural and neurochemical alterations in FSL rats were found to be normalized after chronic treatment with antidepressants (Yadid et al., 2000; Overstreet, 2002). However, whether a high fat diet provokes a depression-like behaviour has never been shown and if that’s the case via which mechanism. On the contrary, HFD consumption has been shown to ameliorate the anxiety and depression symptoms upon an adverse early life environment (Maniam and Morris, 2010a; Prasad and Prasad, 1996).

Vast variations exist in the prevalence of depression across countries suggesting that differences like food habits could account for these differences. It is long believed that mental health and diet are intimately interrelated as demonstrated by the consumption of specific diets and psychiatric disorders. Interestingly, reduced caloric intake from fat and processed food is linked with amelioration in mood and reduced risk for the development of depression in human studies (Akbaraly et al., 2009; Brinkworth et al., 2009). A possible mechanism for the detrimental role of trans fat has been proposed to be that trans-fat lead to the displacement of essential fatty acids from membranes (Innis and King, 1999).

Different categories of fatty acids have been shown to differentially affect the serotonin receptor and transporter binding in the rat brain further suggesting that different dietary patterns might affect the serotonergic signalling (du Bois et al., 2006). Low levels of folate acid have been linked with the development of depression (Gilbody et al., 2007) Folate acid is associated with methylation processes and the synthesis of neurotransmitters in the CNS (Gilbody et al., 2007).
1.19. Fatty acids can cross the blood brain barrier

Lipid molecules are the “building blocks” of the CNS as the mammalian brain is a lipid rich organ, where gray matter contains 36%-40% lipid, white matter 49%-66% and the myelin 78%-81% (O’Brien and Sampson, 1965). However what is the exact molecular mechanism of the transfer of fatty acids from the periphery to the brain remains to be elucidated. The central nervous system (CNS) is protected by the blood-brain-barrier (BBB) composed of brain capillary endothelial cells and invested by astrocytes foot processes (Scherrmann, 2002). The BBB is characterized by enthelial tight junctions and complete absence of pinocytic activity. It regulates the transport of endogenous and exogenous compounds by controlling their selective uptake, efflux and metabolism in and out of the brain (Pardridge, 2002a). Due to the BBB characteristics many therapeutic molecules are precluded the entry in the brain from the blood, thus causing enormous difficulty to the treatment of cerebral diseases. In fact more than 98% small molecules (Tsuji, 2005) and about 100% large molecules are prevented from brain uptake by BBB, including peptides, recombinant proteins, antisense drugs, monoclonal antibodies, and gene therapies including RNA interferes (RNAi).

The plasma levels of free fatty acids (FFAs) are usually 0.2-1.7mM. Fatty acids have been shown to be uptaked and incorporated in the brain (Smith and Nagura, 2001). Circulating macronutrients such as fatty acids can cross the BBB and gain access to the brain where they equilibrate with neuronal LC-CoAs (Miller et al., 1987) (Rapoport, 1996). Several studies have been suggested to explain the fatty acid uptake from the blood into the brain via the capillary membrane. These include 1) receptor mediated transport of acylated species via lipoprotein receptors (Meresse et al., 1989) as well as carrier-mediated uptake of intact lysosphospholipids (Thies et al., 1992; Alberghina et al., 1994; Bernoud et al., 1999). Moreover, fatty acids can be cleaved from the circulating lipids by endothelial lipoprotein lipase for direct influx of free fatty acids (Brecher and Kuan, 1979). Several studies suggest that a primary contributor to brain fatty acid influx is uptake of free fatty acid following dissociation from the plasma protein (Pardridge and Mietus, 1980; Robinson et al., 1992). However, the 99.9% or more of these FFAs are bound tightly with serum albumin (Spector, 1986)
resulting in the concentration of unbound FFAs to range between 0.01-10µM (Spector and Hoak, 1975). Short, medium or long chain fatty acids can cross the BBB, however the mechanism of the transport for the saturated and unsaturated fatty acids has been suggested to be different (Spector, 1988; Avellini et al., 1994). It has already been documented that palmitic acid can cross the BBB (Pardridge and Mietus, 1980). Moreover, palmitic and lignoceric acid can enter the brain from the blood circulation more efficiently than stearic acid does (Morand et al., 1981). The ABC transporters have been shown to play an important role in lipid transport in the brain and their targeting shows beneficial effects for various neurologic diseases (Kim et al., 2008). Under normal physiological conditions the free fraction is estimated to be less than 0.1% in serum (Wosilait and Soler-Argilaga, 1975; Richieri et al., 1993).

Once in the brain the fatty acids can further be metabolized in the mitochondria via β-oxidation, incorporated into phospholipids or affect molecular pathways such as the central control of food intake and energy expenditure (Lopez et al., 2005).

Even though fatty acids are highly hydrophobic and can cross the BBB only two studies focused on the role of fatty acids in crossing the BBB. Myristic acid combined with polyarginine peptide was able to mediate in vivo brain targeted delivery for myristoylated polyarginine in neurons (Pham et al., 2005). In agreement to that, another study showed that molecules conjugated with myristic acid were able to accumulate specifically in the brain after i.v. injections (Meng et al., 2010) suggesting that myristic acid is a potent ligand in the design of brain targeting drug delivery systems. Interestingly, the accumulation of myristic acid in the brain was specific to the hypothalamic area as shown by MRI (Meng et al., 2010) supporting the hypothesis that hypothalamus is a brain region in close proximity and contact with the vasculature. It has been suggested that myristic acid due to the high hydrophobicity is able to incorporate in the phospholipid bilayer of the eukaryotic plasma membrane of the cell is thus capable of acting as a lipid anchor to biomembranes and increasing cellular uptake (Pham et al., 2004; Pham et al., 2005).
1.20. **Main lipids in the CNS**

In contrast to other tissues, the neuronal cell membranes in the CNS are enriched in two main PUFAs: arachidonic acid (20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3) (Sastry, 1985; Diau et al., 2005). Linoleic acid (18:2n-6, the precursor of arachidonic acid) and α-linolenic acid (18:3n-3, the precursor of DHA) exist in plants and are digested and absorbed in the small intestine where they incorporated into the plasma through lacteals. In mammals, they are then being metabolized by elongation and desaturation into arachidonic acid and DHA (Rapoport et al., 2007). The conversion of these precursors into long-chain PUFAs is mostly hepatic although other organs such as the brain possess the necessary enzymes for this conversion (Rapoport et al., 2007). As the synthesis of DHA and arachidonic acid is extremely low in the human body, dietary PUFA are readily incorporated into lipids of the developing brain before and after birth (Innis, 2007) by several types of pathways including the fatty acid translocation system (Coburn et al., 2001), fatty acid binding proteins (Hanhoff et al., 2002; Glatz et al., 2002; Huang et al., 2002), long chain fatty acyl-coA synthetases (Chiu et al., 2001), acyl-coA binding proteins (Knudsen, 1990) and fatty acid transport proteins (Stahl, 2004). The effect of different psychiatric disorders on the function of these enzymes has not been described but any changes in the activity of these enzymes caused under psychiatric disorders might affect their normal translocation and incorporation in the brain.

Long chain PUFAs (LC-PUFAs) are critical components for the neuronal and immune cell membranes and necessary for the normal development, structure and function of the CNS and the prevention of various neurological diseases (Bourre, 2005; Innis, 2003; Salem et al., 2001a; Salem et al., 2001b; Innis, 2007; Su, 2010) and different biological mechanism have been proposed to explain this effect (Bourre, 2006a; Gomez-Pinilla, 2008; Pawels and Volterrani, 2008). Mammals are limited in synthesizing PUFA de novo so they can only obtain them from a balanced diet containing appropriate amounts of these precursors in order to maintain sufficient brain levels of LC-PUFAs in the CNS (Lauritzen et al., 2001; Burdge et al., 2003). Besides their roles as sources for membrane synthesis and energy production, PUFA is believed to be also involved in cell signalling,
gene transcription and neuronal plasticity (Nunez, 1997b; Duplus et al., 2000; Chen and Bazan, 2005).

Aberrations in PUFA composition in cell membranes can alter membrane microstructure, cause abnormal signal transduction and immunologic dysregulation, resulting in increased risk for the development of depression (Horrobin and Bennett, 1999; Logan, 2003). In accordance to that, it has been noted that alterations can occur in the PUFA composition of the neuronal membranes of people with various psychiatric disorders (Su et al., 2000).

DHA is the most abundant ω-3 fatty acid in the mammalian brain and enriched in brain phospholipids (Sastry, 1985) constituting of 30-50% of the weight of a neuron’s plasma membrane. It has been shown to increase during development and to decrease with aging (Youdim et al., 2000; Horrocks and Farooqui, 2004; Innis, 2008). Fish is the main source of DHA. Arachidic acid (AA) is found in the brain and its metabolic products are crucial to orchestrating immunity and inflammation (Calder, 2001).

PUFAs can stimulate the proliferation and differentiation of neuronal progenitors that have been shown to improve neurogenesis and cognitive impairments such as learning and memory or mood disorders as well as the prevention of different neurological diseases. Indeed, PUFA deficiency was related to cognitive impairment (Conquer et al., 2000) and mental disorders such as depression and schizophrenia (Freeman, 2000; Ruxton et al., 2004). More specifically, decreased levels of brain DHA due to nutritional deficiency is associated with reduced cognitive ability, increased emotional behaviour and decreased vision (Rapoport et al., 2007). Severe brain DHA depletion by multigenerational ω-3 PUFA deprivation in rats has been reported to reduce neuronal size in the hypothalamus (Ahmad et al., 2004). In accordance with that, single generational deprivation of dietary ω-3 PUFA results in defective serotonergic and dopaminergic signalling, the main signalling pathways involved in depression (Kodas et al., 2004; Kodas et al., 2002). In accordance, ω-3 PUFAs deprivation postweaning, leads to a reduction in the levels of DHA and increased depression (DeMar et al., 2006). On the contrary, enrichment in endogenous levels of DHA improves spatial learning in the fat-1 transgenic mouse, and this is associated
with increased hippocampal neurogenesis (He et al., 2009). Furthermore supplementation of DHA and ARA in patients with memory impairments, due to stroke or head injuries, results in remarkable improvement in learning and memory (Kotani et al., 2006; Yamashima, 2008). In accordance to the previous studies in the brain, a correlation between specific erythrocyte phospholipid fatty acid composition have given rise to the “phospholipid hypothesis” of depression in the periphery (Hibbeln and Salem, 1995).

Even though there is a relationship between brain PUFA composition affected by dietary modifications and monoaminergic neurotransmission (Delion et al., 1996; Zimmer et al., 2000; Kodas et al., 2004) the exact signalling pathway by which PUFAs conduct their effects on the adult brain still remains poorly understood. However, it has been speculated that the transcription factor, CREB may play and important role because of the increase in its phosphorylation that occurs in rats fed on DHA-enriched diets, which also leads to an improvement in cognitive function (Wu et al., 2008). In contrast to this, rats with PUFA-deprivation exhibit decreased levels of CREB phosphorylation and exhibit a depression phenotype (Rao et al., 2007).

1.2.1. Mechanisms controlled by dietary fatty acids in the CNS

As mentioned previously, western diet is poor in essential fatty acids that are necessary for the brain development and function and characterized by a high ratio of ω-6/ω-3 PUFA (Simopoulos, 2009) and high in trans and saturated fat. Fatty acid composition determines the biophysical properties of neuronal membranes and influences neurotransmission (Salem et al., 2001a). Several biological mechanisms have been suggested to explain the inverse associations between LC- ω-3 PUFA consumption as well as disproportion ratio between ω-3/ω-6 PUFAs as well as the high consumption of trans and saturated fatty acids and the risk for mental disorders. Omega-3 PUFAs are essential components of the neuronal membranes of the CNS and have been implicated in the dynamic structure and changing the membrane fluidity and possibly leading to an enhancement or impairment of the structure and/or function of any membrane/associated receptor complexes. (Horrocks and Farooqui, 2004)
therefore there are many mechanisms by which PUFAs might influence cell function (Marszalek and Lodish, 2005). Higher ω-3 PUFA concentrations lead to increased membrane fluidity, which sequentially increases serotonin transport, a major pathway involved in depression (Hibbeln and Salem, 1995; Mullen and Martin, 1992; Fernstrom, 1999). Due to the fact that the brain has high concentration of LC-PUFAs, neurons are very likely to be sensitive to the ratio of ω-3/ω-6. Therefore, dietary deficiency of ω-3 PUFAs consumption as well as disproportion of ω-6/ω-3 PUFA has been suggested to modify the brain lipid biochemistry, affecting monoaminergic neurotransmitter and glutamate receptors resulting in the impairment of behaviour (Chalon, 2006; Bourre, 2005). Thus, PUFAs play a key role in affecting receptor function, neurotransmitter reuptake resulting in the alteration of the intracellular and intercellular signalling pathways. A possible mechanism that has been suggested to link ω-3 deficiency and the development of a depression phenotype is via the endocannabinoid system (Lafourcade et al., 2011).

1.22. The beneficial role of ω-3 fatty acid intake in depression

Polyunsaturated ω-3 fatty acids (ω-3 PUFA) intake is believed to confer beneficial effects on human health. Food guidelines recommend regular fish consumption in general population to provide a main source of ω-3 PUFAs. A large number of retrospective and prospective studies, as well as clinical trials, revealed that consumption of fish and fish oils rich in ω-3 LC-PUFAs has a lower prevalence of depression (Appleton et al., 2010; Ruxton et al., 2004; Su et al., 2000). This suggests a link between ω-3 PUFAs and the pathogenesis of depression. The SUN cohort, a cohort in Spain characterized by Mediterranean diet showed a potential beneficial effect of omega-3 PUFA intake of total mental disorders (Sanchez-Villegas et al., 2007). There is a 60-fold variation across countries in annual fish consumption and a strong inverse relationship between national consumption of fish per capita and the prevalence of depression (Hibbeln, 2009; Hibbeln, 1998; Samieri et al., 2008). Several epidemiological and clinical data link ω-3 deficiency with neuropsychiatric, mood and neurodegenerative disorders such as depression (Parker et al., 2006a; Hibbeln, 2002; Otto et al., 2003; Llorente et al., 2003; Makrides et al., 2003). Low levels
of PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been detected in the serum and red blood cell membranes of people with depression and this negatively correlated with the severity of depression (Edwards et al., 1998; Adams et al., 1996; Peet et al., 1998). The ratio of ω-6 to ω-3 in erythrocytes and plasma of depressive patients correlated positively with the severity of depression (Adams et al., 1996).

A negative correlation was found between fish consumption and worldwide depression prevalence (Hibbeln, 1998) and, similarly, an inverse association between DHA concentration in human milk and post-partum depression prevalence (Hibbeln, 2002). Association between higher severity of depression symptoms and lower ω-3 fatty acids levels have been found in several studies (Maes et al., 1999; Maes et al., 1996; Parker et al., 2006b).

The beneficial role of ω-3 PUFA on the function of nervous system (Fontani et al., 2005) and on neuropsychiatric alterations treatment (Logan, 2003; Logan, 2004) has been underlined. Clinical trials designed to assess the effectiveness and beneficial effect of ω-3 PUFA supplementation on depression have delivered remarkably favourable results (Nemets et al., 2006; Nemets et al., 2002; Peet and Horrobin, 2002) and to increase the brain serotonin levels (Li et al., 2000). Therefore the effectiveness of antipsychotic drugs when combined with changes in the diet has been suggested to have a faster effect in ameliorating the symptoms of depression. These effects, however, were not the same across all the patient group populations (Hallahan et al., 2007; Peet and Stokes, 2005; Rogers et al., 2008; Freeman et al., 2008). Thus a meta-analytic review of double-blind placebo-controlled trials uncovered the antidepressant effect of ω-3 PUFAs exemplified by a significant improvement in the symptoms of depressed subjects (Lin and Su, 2007; Stahl et al., 2008).

Different trials using fish oils for the treatment of depression showed a significant improvement in the depression scores for both the group that consumed fish oil as well as the control group that received olive oil (Silvers et al., 2005). A possible explanation of the antidepressant effect of both fish and olive oil might be the result of a simple increase in fat intake, as low fat diets can adversely affect mood (Bourre, 2006b; Logan, 2005).
In addition to their beneficial role in mood disorders and depression, diets rich in \( \omega-3 \) fatty acids have been shown to promote weight loss with unaltered energy intake (Hill et al., 2007; Wang et al., 2002). This event has been suggested to be promoted via increased energy expenditure, increased fatty acid oxidation and suppression of the lipogenic pathways (Roche and Gibney, 1999; Sampath and Ntambi, 2005).

### 1.23. Imbalance intake of \( \omega-3 \) and \( \omega-6 \) leads to depression

In contrast to the main human diet in the past, which was focused on hunting and gathering of food rich in \( \omega-3 \) PUFAs, western diet is notoriously poor in these nutrients. Modern diets are accompanied by a large increase in the consumption of vegetable oils, containing \( \omega-6 \) PUFAs, at the expense of \( \omega-3 \) PUFAs (Hallahan and Garland, 2005). The lack of the \( \omega \) essential fatty acids has been linked with many psychiatric disorders such as depression (Perica and Delas, 2011). Particularly, since the dawn of the industrial revolution, which led to an increased intake in processed, high caloric food, the ratio of \( \omega-6/\omega-3 \) PUFAs in the diet has increased from 1 to almost 15 in industrialized countries, leading to a significant deficiency in \( \omega-3 \) PUFAs (Simopoulos, 2002; Simopoulos, 2009). Interestingly the relationship between depression and diet was not caused as a secondary effect due to inflammation, atherosclerosis suggesting the direct effect of fatty acid composition on mood (Tiemeier et al., 2003).

Even though both \( \omega-3 \) and \( \omega-6 \) PUFAs are important for the brain function and development, the lack of \( \omega-3 \) LC-PUFAs or an imbalance between \( \omega-3 \) and \( \omega-6 \) fatty acids resulted from diet rich in linoleic acid has been associated with neurological and psychiatric disorders such as depression in both children and adults (Tiemeier et al., 2003; Wolfe et al., 2009) as well as in mice (Lafourcade et al., 2011). There are many clinical and epidemiological studies that show associations between \( \omega-3/\omega-6 \) imbalance and mood disorders (Parker et al., 2006a). Patients with clinical depression have either an elevated ratio of circulating long chain \( \omega-6/\omega-3 \) PUFAs (Calder, 2001; Peet et al., 1998; Hibbeln, 2009) or just low circulating long chain \( \omega-3 \) PUFAs (Lin et al., 2010). Greater consumption of linoleic acid correlated with higher rates of homicide mortality.
with over a 20-fold change across different countries (Hibbeln et al., 2004). Dietary intake of seed oils, which are rich in linoleic acid, has been associated with depressive symptoms in an elderly (Kyrozis et al., 2009). The elevated levels of linoleic and dihomo-gamma-linoleic acid are the key factors that result in the change of the $\omega-6/\omega-3$ ratio (Chilton et al., 2011). Therefore the dietary $\omega-6/\omega-3$ ratio is of fundamental importance due to their distinct physiological properties and the fact that they compete for their biosynthetic enzymes and any imbalance can lead to mood disorders such as depression. Olive oil which is a main component of the Mediterranean diet, even though it is a vegetable oil, it is rich in oleic acid which is a polyunsaturated $\omega-9$ fatty acid.

1.24. The free fatty acid receptor family

Fatty acids have been shown to act via two major pathways: they can either signal directly by binding to their free fatty acid receptors (Kostenis, 2004) or they can exert a regulatory role via their diffusion in the cell and activate a variety of proteins (Sidhu et al., 2000). There, they can act as substrates to be metabolised and incorporated into intracellular lipid signalling pathways (Warnotte et al., 1994). The free fatty acids act via the binding on their free fatty acid receptors in order to regulate intracellular molecular pathways. High throughput screening (HTS) and ligand fishing deorphanizing strategy successfully identified and assigned three receptors with unknown ligands as receptors for free fatty acids (Hirasawa et al., 2008). These receptors that belong to the rhodopsin family of the GPCRs were previously characterized as a cluster of orphan GPCRs comprising FFAR1 (GPR40), FFAR2 (GPR43) and FFAR3 (GPR41) and are activated by FFAs within physiological concentration ranges (Brown et al., 2005) and with varying degrees of specificity (Briscoe et al., 2003) (Itoh et al., 2003) (Kotarsky et al., 2003) (Hirasawa et al., 2005). The free fatty acid receptor family plays critical roles in a variety of physiological and pathophysiological processes especially in metabolic disorders (Hara et al., 2011). GPR40-43 are located on the human chromosomal locus 19q13.1 (Sawzdargo et al., 1997). GPR40-43 are thought to have evolved relatively recently and they contain several gene duplications, of which GPR41 and GPR42 are examples (Brown et al., 2003). This family of receptors that belong to the
subfamily of nucleotide and lipid receptors shares an overall sequence homology of 30-50% (Brown et al., 2005; Tikhonova et al., 2007) with higher homology within their putative transmembrane domains (Surgand et al., 2006).

GPR41 and GPR43 have been shown to be stimulated by short-chain fatty acids (C1- C6:0), sharply becoming far less responsive to fatty acids of seven or more carbons (Brown et al., 2003; Le Poul et al., 2003). On the contrary, GPR40 prefers fatty acids of medium to long chain length (Briscoe et al., 2003; Itoh et al., 2003) even though it has been documented that higher concentrations of short chain fatty acids can activate GPR40 (Stewart et al., 2006). GPR41 and GPR43 even though they are very similar and bind the same ligands selective orthosteric agonist for GPR43 have been developed suggesting the possibility of the specific targeting of these receptors (Schmidt et al., 2011).

GPR40 is only 30% identical to GPR41 and GPR43 suggesting that its function has clearly diverged from its related receptors (Brown et al., 2003). The putative transmembrane domains of the three receptors share ~39% sequence identity whereas GPR40 is the most dissimilar receptor of the cluster, sharing 35% transmembrane sequence identity with the other members. This sequence differences might confer the chain length specificity of each receptor as GPR40 is the only receptor of the cluster to be selectively activated by medium to long chain fatty acids, whereas GPR41 and GPR43 are activated by short chain fatty acids.

The different pattern of expression of each receptor reflects the different role that has been assigned for the molecular signalling. GPR41 has been shown to play a role in leptin production from adipocytes (Xiong et al., 2004), whereas GPR43 is implicated in differentiation and immune responses of monocytes and granulocytes (Senga et al., 2003). GPR43 has also been shown to be increased in the adipose tissue of obese pigs compare to normal weight (Hou and Sun, 2008). GPR41 is selective for Gi activation, whereas GPR43 can couple and activate Gi and Gq (Brown et al., 2005). Lastly, the absence of selectivity of the different receptors for a single fatty acid implies that selectivity may be determined by the local tissue specific environment.
1.24.1. Other GPCRs that are activated by fatty acids

GPR120 binds to long-chain unsaturated FFAs and is abundantly expressed in lung, intestinal tract, and adipocytes (Hirasawa et al., 2005; Gotoh et al., 2007). Although GPR40 and GPR120 are both activated by long chain FFAs, the two receptors exhibit only 19% sequence identity. GPR120 is involved in the secretion of the glucagon-like peptide-1 from enteroendocrine L-cells (Hirasawa et al., 2005). GPR120 plays a key role as a lipid sensor in the control of energy balance in both rodents and humans (Ichimura et al., 2012).

GPR119 is activated by long chain FFA amid such as oleoylethanolamide and lysophosphatidylcholine (Rayasam et al., 2007; Winzell and Ahren, 2007; Overton et al., 2006) and has been described to coupled with Gs proteins (Soga et al., 2005).

Even though GPR119 and GPR120 share a relatively high sequence similarity, they have a very diverse tissue distribution. GPR40, GPR119, and GPR120 have been described as potential targets for the treatment of diabetes due to their role in regulating the secretion of insulin (Itoh et al., 2003; Briscoe et al., 2006; Hirasawa et al., 2005; Chu et al., 2007; Rayasam et al., 2007). Figure 1.6 shows the distribution of the different fatty acid receptors as well as their main role. As it is shown, their role in the brain is totally unknown.
The free fatty acid receptor 1 (FFAR1) or GPR40 was formally an orphan coupled receptor whose endogenous ligands have been identified as the natural occurring medium to long-chain free fatty acids (FFAs) (Briscoe et al., 2003; Tan et al., 2008) lengths (Brown et al., 2005; Milligan et al., 2006) as well as short chain fatty acids such as butyric in higher concentrations (Stewart et al., 2006) and has attracted considerable attention (Gromada, 2006). At the DNA levels, the rat and mouse GPR40 sequences are 94% identical to each other and 75 and 76% identical to the human GPR40, respectively. At the protein level, rat and mouse GPR40 are 95% identical to each other and 82 and 83% identical to the human receptor, respectively. This highly conserved receptor sequence between mouse, rat, hamster and monkey suggests the important role that plays in the cells as any evolutionally change can compromise its action. It is abundantly expressed on pancreatic β-cells (Tomita et al., 2006; Bartoo-Shifman et al., 2007) and in the intestine and endocrine cells of the gastrointestinal tract (Edfalk et al., 2008) as well as hepatocytes (Suh et al., 2008; Wu et al., 2012) and enhances
free fatty acid glucose-stimulated insulin secretion (GSIS) (Itoh et al., 2003; Briscoe et al., 2003; Kotarsky et al., 2003; Salehi et al., 2005; Nolan et al., 2006). Knock down of GPR40 studies using small interfering RNAs or antisense treatment greatly reduces free fatty acid mediated insulin release (Itoh et al., 2003; Shapiro et al., 2005; Salehi et al., 2005). GPR40 deficient beta cells secrete less insulin in response to FFAs, and loss of GPR40 protects mice from obesity induced hyperinsulinemia, hepatic steatosis, hypertriglyceridemia, increased hepatic glucose output, hyperglycemia and glucose intolerance after HFD (Steneberg et al., 2005). The ob/ob mouse exhibits β-cell hyperplasia due to the increased demand for insulin (Coleman, 1978) and has been characterized by increased pancreatic expression of GPR40 mRNA (Lee et al., 2003; Briscoe et al., 2003), whereas polymorphisms so far detected in the human GPR40 gene are not associated with abnormal insulin release (Hamid et al., 2005). Due to its role in insulin secretion GPR40 has attract considerable attention as a new potential target for the treatment of type 2 diabetes. However, it is unclear whether an agonist or an antagonist (Kebede et al., 2008; Zhang et al., 2010) represents the best therapeutic approach as GPR40 ligand for the treatment of diabetes (Brownlie et al., 2008) as it has been shown that GPR40 knockout and in vivo overexpression mediates both acute stimulatory and chronic detrimental effects of insulin secretion of long-chain free fatty acids on murine insulin secretion (Steneberg et al., 2005). Therefore, many ligands for GPR40 have been discovered as potential novel therapies for obesity and type two diabetes (Christiansen et al., 2012; Tsujihata et al., 2011; Briscoe et al., 2006; Song et al., 2007; Humphries et al., 2009) and different mechanisms of action of these ligands have been proposed (Ou et al., 2013a).

In addition to the FFAs, GPR40 has been reported to be activated by anti-diabetic drugs such as thiazolidinediones (TZD) ligands of the peroxisome proliferator-activated receptor (PPARγ) (Kotarsky et al., 2003; Briscoe et al., 2003) and experimental anti-obesity drugs (e.g., MEDICA 16) (Kotarsky et al., 2003) can activate GPR40.

GPR40 is a Gq-coupled receptor that leads to insulin secretion via the activation of the PLC pathway and the hydrolysis of inositol lipids as well as the intracellular calcium levels (Shapiro et al., 2005; Kotarsky et al., 2003; Fujiwara
et al., 2005; Salehi et al., 2005; Itoh et al., 2003) with subsequent increases in cytoplasmic free calcium (Briscoe et al., 2003; Itoh et al., 2003). Binding of oleic acid on GPR40 induces Ca\(^{2+}\) signalling that is mediated by PLC (Fujiwara et al., 2005). Even though most of the literature has shown that GPR40 is a Gq-coupled receptor it has also been reported as a Gi-coupled receptor leading to the downregulation of the cAMP signalling (Yonezawa et al., 2004; Itoh et al., 2003). It has also been reported to inhibit the activity of potassium channels via a cAMP-dependent pathway (Feng et al., 2006). The binding potencies of medium to long chain fatty acid range from 2 to 17\(\mu\)M (Briscoe et al., 2003). GW9508 is a potent agonist that has been developed for GPR40 (Garrido et al., 2006; Briscoe et al., 2006) and the specific residues from GPR40 that interact with GW9508 have also been identified (Sum et al., 2007). GW1100 is a potent antagonist that has been developed for GPR40 (Briscoe et al., 2006).

Real time PCR analysis has revealed high levels of the GPR40 in pancreas and even higher in the brain with very high levels of detection in the hypothalamus (Briscoe et al., 2003). Due to GPR40’s role in insulin secretion it has been extensively studied in the pancreas despite its widely distribution in the CNS (Ma et al., 2007; Ma et al., 2010). Therefore, even though the high levels of GPR40 expression in the brain its functions in the brain still remain unknown.
### Table 1.2. A family of free fatty acid receptors. GPCRs known to bind fatty acids and their properties.

<table>
<thead>
<tr>
<th>GPCR</th>
<th>Ligands</th>
<th>G protein signaling</th>
<th>Effector</th>
<th>Main expression</th>
<th>Function</th>
<th>Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPR41, FFAR3</td>
<td>SCFAs (C3-C7), formate, acetate, propionate, butyrate, pentanoate</td>
<td>Gi/o, β-arrestin2, Gai/o</td>
<td>↓ cAMP</td>
<td>adipocytes, enteroendocrine L cells</td>
<td>DC maturation, leptin production, regulation of energy balance</td>
<td>(Samuel et al., 2008) (Xiong et al., 2004)</td>
</tr>
<tr>
<td>GPR43, FFAR2</td>
<td>SCFAs (C2-C7), formate, acetate, propionate, butyrate, pentanoate</td>
<td>Gi/o, Gq, β-arrestin2, Gaq/11</td>
<td>↓ cAMP, ↑ Ca2+</td>
<td>innate immune cells, gut epithelium, white adipose tissue, adipocyte, immune cells</td>
<td>gut homeostasis, tumor suppressor, insulin-mediated fat accumulation, control of body energy</td>
<td>(Kimura et al., 2013) (Maslowski et al., 2009)</td>
</tr>
<tr>
<td>GPR109A</td>
<td>SCFAs (C4-C8), Butyrate, nicotinic acid (niacin)</td>
<td>Gi/o, β-arrestin2</td>
<td>↓ cAMP, ↑ Ca2+</td>
<td>adipocytes, neutrophils, macrophages, intestinal epithelial cells</td>
<td>DC trafficking, gut homeostasis, tumor suppressor, intracellular triglyceride, lipolysis in adipocytes</td>
<td>(Lukasova et al., 2011) (Singh et al., 2014)</td>
</tr>
<tr>
<td>GPR120 (FFAR4)</td>
<td>long chain fatty acids (C14-C18), omega-3 fatty acids C14 C22, EPA, DHA, palmitoleic acid, α-linolenic acid</td>
<td>Gq/11, β-arrestin2</td>
<td>↑ Ca2+</td>
<td>enteroendocrine cells in the colon, macrophages</td>
<td>Inhibition of TNF and IL-6, regulation of insulin secretion by GLP-1</td>
<td>(Hirasawa et al., 2005) (Ichimura et al., 2012) (Oh et al., 2010)</td>
</tr>
<tr>
<td>GPR40</td>
<td>medium to long chain fatty acids (C12-C18) Thiazolidinedione</td>
<td>Gq/11</td>
<td>↑ Ca²⁺</td>
<td>pancreatic beta cells</td>
<td>enteroendocrine K cells</td>
<td>insulin secretion of pancreatic beta cells</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------------------------------</td>
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<td>-----------------------</td>
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<td>---------------------------------------------</td>
</tr>
<tr>
<td>GPR84</td>
<td>medium chain fatty acids (C9-C14) capric acid, undecanoic acid and lauric acid</td>
<td>Gi/o</td>
<td>↓ cAMP</td>
<td>immune cells</td>
<td>spleen monocytes</td>
<td>GPR35 SNPs implicate in human inflammatory bowel disease (Suzuki et al., 2013)</td>
</tr>
<tr>
<td>GPR35</td>
<td>Kynurenic acid, kysophosphatidic acid, pamoic acid, β-arrestin2</td>
<td>Gi/o</td>
<td>↓ cAMP</td>
<td>monocytes neutrophils</td>
<td>monocytes iNKT cells, GI tract peripheral nervous tissues, mast cells</td>
<td>GPR35 SNPs implicate in human inflammatory bowel disease (Suzuki et al., 2013)</td>
</tr>
<tr>
<td>GPR91</td>
<td>succinate</td>
<td>Gi/o</td>
<td>↓ cAMP</td>
<td>high levels in adipose tissue kidney, nervous system, DCs, lower levels in liver, spleen</td>
<td>proinflammatory, migration of Langerhans cells, hematopoiesis, angiogenesis, hypertensive effects, activation of renin-angiotensin system (Sapieha et al., 2008) (Toma et al., 2008)</td>
<td></td>
</tr>
<tr>
<td>GPR119</td>
<td>Oleoylethanolamide, lysophosphatidylcholine, Oleoylethanolamine and N-oleoyldopamine</td>
<td>Gas</td>
<td></td>
<td>B cell entero-endocrine cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPR132</td>
<td>9-hydroxyoctadecadienoic acid</td>
<td>Gaq/11</td>
<td></td>
<td>Macrophages</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Fujita et al., 2011) (Itoh et al., 2003)
1.25. Cellular lipotoxicity due to fatty acid exposure

Lipotoxicity is a metabolic stress response that has been shown to involve lipid-induced oxidative stress. It involves a series of pathological cellular responses after exposure to elevated levels of fatty acids (Unger, 2003). Lipotoxicity is one of the main mechanisms that have been involved with the decline and damage of the β-pancreatic islets that leads to diabetes (Robertson et al., 2004). Hyperlipidemia which is the excessive delivery of fatty acids to non-adipose tissues results in lipid accumulation and is associated with cellular dysfunction and cell death through lipotoxicity (Unger, 1995). It has been studied in many different organs and cell types but the cellular and molecular mechanisms underlying this process are totally unknown. Moreover, very few studies were done on neuronal cells to examine the role of lipotoxicity in neurodegeneration.

Different cell systems employing fibroblast, myoblasts, hepatocytes and other cell lines have been used in order to characterize the cellular mechanisms underlying lipotoxicity. In these models, the growth media is supplemented with excess of fatty acids complexed to albumin in order to achieve pathophysiological ranges of free fatty acids. These studies demonstrated a time and dose dependent induction of cell death that is characterized by features of apoptosis (Cacicedo et al., 2005; de Vries et al., 1997; Listenberger et al., 2003; Wei et al., 2006).

An important and interesting neurochemical event initiated by traumatic brain injury or hypoxic-ischemic insults in the CNS is the degradation of membrane phospholipids accompanied by the release of high levels of FFAs (Bazan, 1970; Rehncrona et al., 1982; Zhang and Sun, 1995; Dhillon et al., 1997; Homayoun et al., 1997). FFAs are liberated from the lipid membrane by intracellular lipases (Abe et al., 1987; Faden et al., 1987) and may act as the mediators of the secondary damage (White et al., 2000) further contributing to the pathogenesis of lipotoxicity (Lee et al., 1994). The elevation of FFAs in these pathophysiological conditions consists mainly of palmitic acid, stearic acid, oleic acid and smaller amounts of arachidonic acid and docosahexaenoic acid (Lukacova et al., 1998). Arachidonic acid has been shown to form toxic...
metabolites therefore it is suggested to be a possible mediator of FFA mediate injury on the CNS.

Interestingly, the pathological effects of the different fatty acids are not the same between the different fatty acid categories. The lipotoxicity response is relatively specific for the saturated free fatty acids and is increased by simultaneous exposure to elevated glucose concentrations (El-Assaad et al., 2003). Exposure to high levels of palmitic acid exhibits significant lipotoxicity and apoptotic cell death of cortical neurons and PC12 cells which is a neuronal cell line (Almaguel et al., 2009). Moreover, reports have demonstrated that the ability of fatty acids to cause apoptosis in the rat pancreatic cell line and in human islets is dependent on the degree of saturation. Even though stearic and palmitic acid have been shown to induce a pro-apoptotic and toxic effect the oleic and arachidonic acid do not have this effect in vitro in concentrations that are similar to the ones after CNS injury (Ulloth et al., 2003). Moreover, oleic, palmitoleic and linoleic have been shown to have a protective, non-apoptotic role of ß-cells when they are being incubated with saturated fatty acids (Eitel et al., 2002; Maedler et al., 2003).

The pleiotropic effects of FFAs on the pancreatic ß-cell function are well known. FFAs bind on GPR40 and stimulate insulin release (Prentki et al., 1992; Warnotte et al., 1994). However, FFAs also exert a suppressive or detrimental effect on ß-cells via GPR40 (Steneberg et al., 2005). Lipotoxicity of ß-cells due to chronic exposure to high FFA levels, results in impairment in their function and a resulting diminution in their insulin secretory capacity (Steneberg et al., 2005).

Obesity is a disorder characterized by metabolic abnormalities such as elevated levels of circulating free fatty acids due to unrestrained lipolysis with ensuing detrimental effect on the ß-cell function both in rodents and humans (Carpentier et al., 2000; Sako and Grill, 1990). However, the pathological effect of this fatty acid overload in obesity on the brain function is unknown. Interestingly, it has been observed that people with surgeries to remove part of their adipose tissue claim that their mood improves immediately. This suggests that the circulating fatty acids due to the high amounts of adipose tissue can get
access in the brain and regulate signalling pathways responsible for the depression phenotype rather than the actual body weight.

1.26. Main hypothesis and thesis aims

Depression is a disorder with high socioeconomic burden for the society and the individual. For instance, a link between obesity and depression was first suggested by different epidemiological and clinical studies (Zhong et al., 2010; Leonore de Wit and Frans Zitman, 2010; Boutelle et al., 2010; Stunkard et al., 2003; Simon et al., 2010; McElroy et al., 2004; Faith et al., 2002; Luppino et al., 2010; Palinkas et al., 1996; Roberts et al., 2003; Dong et al., 2004; Roberts et al., 2002). Moreover, the higher the BMI of the individual the stronger the relationship with depression (Onyike et al., 2003; Boutelle et al., 2010). Given the prevalence of overweight and obesity in the western world and their rapid increase in developing countries even a modest increase in depression represents a major public health problem. However, even though epidemiological studies are particularly effective for the identification of the link between obesity and depression but rarely identify causative relationship or elucidate the underlying mechanisms.

There are several endogenous pathways that have been involved in depression; one of them is the cAMP pathway (Duman et al., 1997). cAMP is an important second messenger that can regulate a wide spectrum of physiological processes in the cell. It is believed that current antidepressant treatments act via the up-regulation of different molecules at the cAMP pathway (Duman et al., 1997). Obesity and depression are both very multicomponent disorders and cAMP is an attractive target as it can orchestrate pathways in both disorders. Among patients with depression and obesity the heterogeneities in lifestyles and diets make it difficult to make it difficult to design experiments that uncover causality or mechanistic links between these complex diseases. During the past 40 years intense research has been focused on the different neurotransmitters for the development of antidepressant drugs (Nestler et al., 2002a). However, 50% of patients do not response to antidepressants (Berton and Nestler, 2006). In some of these studies it was pointed out that being overweight or obese is a risk factor for resistant to antidepressant treatment (Papakostas et al., 2005). These
observations implicate the involvement of unique pathways for the obese and overweight population that lead to the development of depression and are not targets of the common antidepressants.

Our main hypothesis proposes that dietary or genetic obesity can be a causative factor for the development of depression. Through mRNA array analysis as well as *in vivo* and *in vitro* experiments we will then try to identify the molecular pathway of this phenomenon. The identification of novel molecules involved in depression will help the development of new therapeutic targets for depression specific for individuals that show a resistant to current antidepressants such as the overweight and obese.

First, in order to test this hypothesis we wanted to determine whether either genetic or dietary obesity can be a causative factor for the development of depression as a causative role has not been fully established from the various studies. In order to do this we utilized commonly used behavioural paradigms to test for depression. We were able to identify a causative role of obesity either genetic or dietary for the development of depression. Interestingly, this effect was independent of the increase in the body weight caused by either the genetic or dietary obesity. Next, mRNA array comparative analysis which is a powerful technique to identify signalling pathways and specific molecules that are affected was performed between normal mice and mice that developed depression. We were able to implicate the PKA signalling cascade in the hypothalamus responsible for the depression phenotype due to the consumption of a HFD.

Secondly, we wanted to further investigate the molecular mechanism of the dietary obesity induced depression phenotype. PKA activity is regulated by cAMP levels and the sole route for the cAMP degradation happens via a large family of molecules the PDEs. We were able to identify a specific PDE4 phosphodiesterase isoform, namely PDE4A5, to play an important role for the development of dietary and genetic obesity-induced depression as the *in vivo* loss of PDE4A rescues the depression phenotype in both types of obesity. Even though many studies have shown the protective role of polyunsaturated fatty acids on
depression little is known about the negative role of specific dietary components in mood. Paradoxically the detrimental role of other fatty acids that can play a causative role for the development of depression has not been studied. In this study we were able to identify palmitic acid, which is a major fatty acid in the HFD to affect the PKA signalling. Our preliminary data suggest a potential direct role in inducing a depression phenotype. Finally, we were able to identify a free fatty acid receptor (GPR40) as a novel binding partner of PDE4A5 that is upregulated in the hypothalamus and is responsible for the depression phenotype after the consumption of HFD. By blocking the activation of this receptor in the hypothalamus using a specific GPR40 antagonist we were able to partially rescue the dietary obesity induced depression phenotype observed. This receptor would represent a novel therapeutic target for the treatment of depression that is caused by dietary factors.

Therefore the main aims in this thesis are as follows:

1) To address whether obesity in mice can play a causative role for the development of depression phenotype

2) To determine the molecular pathways in the brain that are altered after the dietary induced obesity phenotype
Chapter 2

Materials & Methods
Chapter 2  Materials & Methods

2. Materials

All chemicals used in this study were of analytical grade. All compounds were supplied by Sigma-Aldrich, USA and fatty acids were purchased from Nu-Check, USA and they were typically dissolved in dimethyl sulfoxide (DMSO) unless otherwise stated. All aqueous solutions were prepared using water purification system with automatic sanitization (Millipore, USA).

2.1. Animal procedures

2.1.1. Animals

WT C57BL/6 mice and leptin deficiency mice (Lep$^{ob}$ or ob/ob) were obtained from The Jackson Laboratory and bred in the mouse facility at the Gladstone Institutes, University of California San Francisco (UCSF). The PDE4A$^{-/-}$ mouse line was a gift by Professor Marco Conti that he generated and crossed with a C57BL/6 background (11 or 12 crossings). The generation of the PDE4A$^{-/-}$ was done and described by Professor Marco Conti (Jin and Conti, 2002; Jin et al., 2005) (Jin et al., 1999). All mice were bred in house, maintained under constant temperature (23 $^\circ$C) and lighting (lights on from 0700-1900 h) and were allowed free access to food pellets and water. Following mating, litters were housed with the dam in polypropylene cages (20 cm x 27 cm x 17 cm) with wood shavings and a metal lid in groups of 5 mice. All studies were done using male mice that had access to food and water ad libitum. All animal study protocols and procedures were reviewed and conducted in accordance with the Guide for the care and use of laboratory animals (LARC) at UCSF and approved by the institutional animal care and use committees of UCSF, and are in compliance with standards set by the National Institutes of Health.
2.1.2. Genotyping

Newborn pups up to 7 days old were toe clipped for identification purposes. A small portion of the tail was taken for DNA isolation and genotyping purposes. Each tail is digested in 500 µl PCR lysis buffer (100 mM Tris pH 8.5, 5 mM EDTA pH 8.0, 200 mM NaCl and 0.2% SDS) supplemented with 4 µl of recombinant protein Proteinase K of 14 - 22 µg/µl (Roche). The digestion was incubated at 150 rpm overnight at 55 °C. The digestion product was spun down for 10 minutes at maximum speed to separate the tail hair from the DNA. The supernatant was then transfected to another tube with 500 µl of isoprotenerol to precipitate the DNA. DNA is insoluble in isoprotenerol and will fall out of solution fast. The precipitated DNA was then transferred with a tip in a new tube with ultrapure protease and nuclease free water. Polymerase chain reaction (PCR) was performed using the quick load master mix (England Biolabs) to determine the genotype of the mouse. In this study two different PCRs were performed for genotyping purposes. The sequences of the primers are shown in the Table 2.1.

Table 2.1. Sequences of the primers used for genotyping.
The first three primer sequences are used for the genotyping of the PDE4A. The last two primers are used for the genotyping of leptin.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDEA4(4A-5F)</td>
<td>GATTCTCAGACTGTGGCACC</td>
</tr>
<tr>
<td>PDE4A(4A-8R)</td>
<td>GCAGGTCAGGCGCTTACAC</td>
</tr>
<tr>
<td>PDE4A(KO2F)</td>
<td>CTAAGGCGCATGCCTCCAGACTG</td>
</tr>
<tr>
<td>leptin1151</td>
<td>TGTCCAAGATGGACCAGACTC</td>
</tr>
<tr>
<td>leptin1152</td>
<td>ACTGGTCTGAGGCAGGAGCA</td>
</tr>
</tbody>
</table>
PDE4A PCR program

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>repeat next 3 steps for 35 cycles</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>30''</td>
</tr>
<tr>
<td>Annealing</td>
<td>59 °C</td>
<td>30''</td>
</tr>
<tr>
<td>Elongation</td>
<td>72 °C</td>
<td>1'15''</td>
</tr>
<tr>
<td>Final Elongation</td>
<td>72 °C</td>
<td>10'</td>
</tr>
</tbody>
</table>

The 3 PCR primers for the PDE4A genotyping shown in Table 1 [PDE4A(4A-5F), PDE4A(4A-8R) PDE4A(KO2F)] are used in combination in the same PCR in order to produce 2 different PCR fragments. The WT produces a fragment of 780 b.p. and the PDE4A^-/- mouse produces a fragment of 423 b.p.

Leptin PCR program

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>repeat next 3 steps for 35 cycles</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>30''</td>
</tr>
<tr>
<td>Annealing</td>
<td>62 °C</td>
<td>1'</td>
</tr>
<tr>
<td>Elongation</td>
<td>72 °C</td>
<td>45''</td>
</tr>
<tr>
<td>Final Elongation</td>
<td>72 °C</td>
<td>10'</td>
</tr>
</tbody>
</table>

The two PCR primers (leptin1151 and leptin1152) lead to a fragment that is further digested with Ddel restriction enzyme (England Biolabs) for 4 hours at 37 °C. The WT gives a band of 155 b.p. and the mutant a band of 55 b.p. and 100 b.p.

The analysis of the PCR fragment was performed by agarose gel electrophoresis in order to separate DNA by molecular weight. The PCR reaction is already loaded with a running dye from the PCR master mix (England Biolabs). Agarose gel was prepared with tris-acetate acid-EDTA (TAE) buffer containing 40 mM Tris-Cl; pH 8.5, 0.114% (v/v) glacial acetic acid and 2 mM EDTA. The agarose solution was allowed to cool slightly before adding 1 µl/100 ml of ethidium
bromide. For gel casting, the solution was poured into the Bio-Rad Mini-Sub Cell GT agarose gel system with comb inserted to create wells and allowed to solidify at room temperature. A 1.5% agarose gel was prepared for the separation of the PDE4A PCR and a 2.5% agarose gel for the separation of the leptin PCR. Once the gel was polymerized the comb was removed and the gel was placed in a gel tank containing 1xTAE buffer. A 1 kb DNA ladder (England Biolabs) was used as a molecular size marker. The gel was run at 80 V until the dye has migrated about 2/3 of the gel. Negatively charged DNA samples will migrate from the cathode toward the anode during electrophoresis. The gel was then removed from the tank and visualized on an ultraviolet transilluminator (Gel Doc XR+ System, Bio-Rad).

2.1.3. Diets

Mice on normal diet (ND) were fed the PicoLab Rodent Diet 20 (catalog # 5053) from LabDiet that consists of 13.2% fat, 62.1% carbohydrates and 24.7% proteins. Mice on the high fat diet (HFD) were fed a 60% fat content diet from Research Diets (catalog # 12492) with 20% carbohydrates and 20% proteins. Experiments in this study were performed in mice following either 3 or 8 weeks on the two different diets. 3 weeks represents a time point before the development of metabolic syndrome and 8 weeks is after the development of metabolic syndrome (Hwang et al., 2010).

2.2. Behavioural assays

Behavioural tests were conducted in order to assess the depression phenotype in mice. All behavioural tests were conducted during the light phase (1200-1500 h) and measurements commenced 4 h after lights on. Animals were allowed to adapt to the experimental room for at least 1 h before testing. The mice were tested according to the following sequence of tests: open field, elevated plus maze, sucrose preference test, tail suspension test and forced swim test placing the most stressful tests in the end.
2.2.1. Open field test

To assess the total locomotor activity, the open field test was used. This test is commonly used to quantitative and qualitative measure the general locomotor activity as well as the exploratory behaviour of rodents. The test was carried out using a Digiscan locomotor activity monitor (Model RXYZCM, Omnitech Electronics; Columbus, OH) which consists of an open-top 40cm (L) x 30cm (H) clear Plexiglass holding box equipped with photocell beam sensor bars (16 infrared sensors per bar, 2.5cm apart) running horizontally across the walls (50). Each mouse was placed in the middle of the box for 15 minutes and a computer tabulated its movements automatically. The holding box was cleaned with 70% ethanol before receiving another mouse.

2.2.2. Tail suspension test

This test has been used to assess the effectiveness of antidepressant drugs in rodents and generally reduced immobilization time is an indicator of an antidepressant effect (Cryan et al., 2005), (Cryan et al., 2002a) (Nestler et al., 2002b). Mice were subjected to the tail suspension procedure by hanging them by their tails in a tail suspension system (35 cm x 30 cm), as has been previously described (Cryan et al., 2005). Briefly, mice were hung via a strip of masking tape with their nose positioned ≈350 mm from the floor for 6 min. Behaviour was monitored and recorded using a camera for the last 4 minutes. Immobility,
defined as the summation of time mouse does not struggle to escape was quantified 3 times and averaged. Experimenter was present during the video capture.

2.2.3. Forced swim test

To assess helplessness, the Porsolt forced swim test was used to assess for depressive-like behaviour in rodents (Porsolt et al., 1977b) (Porsolt et al., 2001; Cryan et al., 2002b). This test counts rodent’s response to threat of drowning and has been widely used to measure the effectiveness of antidepressants. The time spent floating represents the immobilization time. Mice were individually placed in an inescapable transparent Plexiglas cylindrical tank (40 cm high x 20 cm in diameter with a depth of 30 cm) filled to a depth 25 cm with tap water (25 ± 2 °C) for 6 minutes, and behaviour was recorded with a camera for the last 4 minutes. Immobility, considered the absence of mobility other than the minimal necessary for the mouse to stay afloat, was scored 3 times and averaged. Experimenter was present during video capture.

![Figure 2.3. Forced swim test. Mouse is placed in a cylinder with water and immobility time is counted.](image)

2.2.4. Sucrose preference test

Anhedonia was tested using sucrose preference test (SPT). To do this, mice were singly housed and habituated with ad libitum food and two bottles - one with tap water and one with 2 % sucrose solution, for 3 days prior to experimental start. Bottles were reversed every day throughout the time of the habituation to avoid side preference. On the day of the test, mice were deprived from both water and sucrose for 8 h. At the end of the day, the two bottles were put back
for 2 h. Bottles were placed in a different order in every cage to avoid side preference. The consumption of water and sucrose solution was estimated simultaneously in control and experimental groups by weighing the bottles. The preference for sucrose was calculated as a percentage of total liquid consumed (Akinfiresoye and Tizabi, 2013). Sucrose preference below 65 % is usually taken as the criterion for anhedonia, and is based on the ≥ 65 % sucrose preference of control animals (Moreau et al., 1992).

2.2.5. Elevated plus maze (EPZ)

EPM is a commonly employed behavioural animal anxiety test for the measurement of anxiolytic or anxiogenic behaviours in rodents. The model is based on rodents' aversion of open spaces (Hogg, 1996) (Rodgers and Dalvi, 1997). This aversion leads to the behaviour termed thigmotaxis, which involves avoidance of open areas by confining movements to enclosed spaces or to the edges of a bounded space therefore this translates into the restriction of movements to the enclosed arms. Anxiety reduction in the EPM is indicated by an increase in the proportion of time spent in the open arms (time in open arms/total time in open or closed arms), and an increase in the proportion of entries into the open arms (entries into open arms/total entries into open or closed arms). Total number of arm entries and number of closed-arm entries usually represent the general activity. It was conducted after 3 weeks on HFD using a plus shaped maze made of opaque Perspex with two opposite open arms (50 cm x 10 cm) and two closed arms (50 cm x 10 cm with 40 cm walls) elevated 80cm above the ground. The light intensity was 80 lux. In a 10-min trial, the time spent in the open and closed arms was measured.
Chapter 2  Materials & Methods

2.3. Drug administration

2.3.1. Acute administration of rolipram

Rolipram is a generic PDE4 inhibitor that has been shown to have antidepressant action in mice (Wachtel, 1982) (Wachtel, 1983b) and humans (Bobon et al., 1988) (Wachtel and Schneider, 1986) (Fleischhacker et al., 1992). 9 mice were divided into two groups. The mice received 3 intraperitoneal (i.p.) injections: 2 injections were done the day before (one in the morning and one in the evening) and 1 injection was performed 1 h prior to the tail suspension test. Rolipram was first dissolved in dimethyl sulfoxide (DMSO) and then in saline with the final i.p. concentration 10 mg/kg and 3% final DMSO concentration.

2.3.2. Chronic administration of rolipram and fluoxetine

15 mice were divided into 3 groups and received either 10 mg/kg of fluoxetine (Sigma-Aldrich F132, also known by the trade name Prozac), 1.25 mg/kg of rolipram (Sigma R6520) or normal saline daily for 3 weeks. Both rolipram and fluoxetine were dissolved in DMSO and the same percentage of DMSO was added to the saline control. Rolipram at this dose has been described in preclinical studies to be sufficient to exert an antidepressant effect in rats (O’Donnell, 1993) (Przegalinski and Bigajska, 1983). The behavioural effect of chronic rolipram or Prozac administration was tested after three weeks. All the behavioural tests were performed 1 h after drug injections following the treatment schedule. 24 h after the last injection, mice were killed by decapitation and their hypothalamus, cortex, hippocampus and amygdala were dissected, isolated and immediately frozen in liquid nitrogen.

Figure 2.5. Elevated plus maze. Mouse was placed in the middle of the apparatus and was left to explore for 10 minutes. Picture taken from http://stockholmbrain.se/sbi-research/translational-behavioural-neuroscience-tbn/elevated-plus-maze/
2.3.3. Oral gavage of olive oil and ghee

Oral gavage was performed in wild-type 10 week old mice. The mice were gavaged with 300 µl of olive oil or ghee (clarified butter), two times per day for 7 weeks. Ghee and olive oil were pre-warmed at 37°C for 30 minutes before being administrated in mice. Mice were provided food *ad libitum* at all times. After the completion of the 7 weeks, mice were tested on different behavioural paradigms.

2.3.4. Intracerebrovascular (i.c.v.) administration of GPR40 antagonist via implantation of osmotic pumps

Mice were anesthetized with avertin and placed into a stereotaxic instrument. Avertin was used as anesthesia as it has been shown that there is a possible interaction between xylazine/ketamine anesthesia and PDE4 (Robichaud et al., 2002). Ketamine has also been shown to have an antidepressant action (Salvadore and Singh, 2013). Mice were kept isothermal with heating pads and the surgical site prepared with betadine. Eyes were protected with lubricant. Using aseptic techniques, a 7-mm incision was made through the scalp skin. A pocket for placement of the osmotic pump was created by undermining the skin and the interior of the pocket. Coordinates for the 3rd ventricle were obtained using the Paxinos and Watson coordinates relative to bregma: 1.3 mm bregma, 0.0 mm lateral, and 5.0 mm dorsal-ventral. A hole was drilled through the skull using a dental drill for the cannula implantation. A sterile 28 gauge stainless steel cannula (Alzet, Cupertino, CA) was lowered into the brain to the desired depth (5.0 mm) and secured with acrylic dental cement and metabond (C&B metabond kit #S380). The i.c.v. cannula was connected to an appropriate Alzet osmotic mini-pump containing either 0.1µg/µl or 1 µg/µl GPR40 antagonist (1002 Alzet, Cupertino, CA) via polyvinylchloride tubing. All intracerebroventricular (i.c.v.) solutions were dissolved in artificial cerebral spinal fluid (acsf). The scalp incision was closed with sutures and the mouse was kept warm during recovery. Animals were individually housed and monitored. Stable daily food intake and recovery of pre-operative body weight were used as measures of surgical recovery and typically normalized within 2 to 3 days post-surgery. At the end of
the 14 days of administration, mice were subjected to the tail suspension test. The next day, mice were sacrificed and proper infusion was confirmed in all animals by taking out and visually inspecting the fluid inside of the pump.

2.3.5. Single stereotactic injections of palmitic acid

Mice were anesthetized with avertin and placed into a stereotaxic instrument with ear bars and nosepiece. Avertin was used as anesthesia as it has been shown that there is a possible interaction between xylazine/ketamine anesthesia and PDE4 (Robichaud et al., 2002). Ketamine has also been shown to have an antidepressant action (Salvadore and Singh, 2013). Mice were kept isothermal with heating pads and the surgical site prepared with betadine. Eyes were protected with lubricant. Using aseptic techniques, a 7-mm incision was made through the scalp skin. Coordinates for the hypothalamic area are: -1.5 mm bregma, ±0.4mm lateral, and -5.8 mm dorsal-ventral (Paxinos and Watson 1998). A hole was drilled through the skull using a dental drill for the needle. A sterile 33 gauge stainless steel needle (Hamilton #7803-05) was lowered into the brain to the desired depth (5.8 mm). A 10 µl glass syringe (Hamilton # 7635-01) was attached to the needle. A stock of 250 µg/µl of palmitic acid in 100% ethanol was prepared and further diluted to 1/50 in PBS prior to the in vivo injection, so that the final concentration was 5 µg/µl. Mice received 2 µl per hemisphere, which is 10 µg of palmitic acid in total. As a control, aCSF injected mice were used as well as 1/50 dilution of ethanol in PBS. The 2 µl injection in the hypothalamus was given over a period of 8 minutes (0.25 µl per minute). After the termination of the injection, the needle stayed at the injection site for 5 more min before withdrawing; then the needle was withdrawn slowly to avoid backflow of the solution to the surface. The scalp incision was closed with sutures and the mouse was kept warm during recovery. Animals were individually housed and monitored. Tail suspension test was performed 4 days after the single stereotaxic injections. The following day, the mice performed the open field test. Six days after the injection, mice were sacrificed and the proper injection site was confirmed in all animals by visually checking the hole on the brain.
2.4. **Tissue collection**

At the end of the experiments the mice were placed in a CO\textsubscript{2} chamber and decapitated post-mortem. The isolation was between 1200-1500h. The hypothalamus was removed using brain slicer matrix (Zivic instruments) and was extracted within 1 minute, snap frozen in liquid nitrogen, and stored at -80 °C until further analysis.

2.5. **Preparation of lysates**

2.5.1. **Whole brain lysates**

Brain protein extracts were prepared from hypothalamic, amygdala, cortex and hippocampal samples of WT and PDE4A\textsuperscript{+/−} mice. Brain extracts were lysed in the TNE buffer (10 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP40) or HKEM buffer (50 mM KCl, 50 mM HEPES, KOH Ph7.2, 10 mM EGTA, 1.92 mM MgCl\textsubscript{2} ) supplemented with protease and phosphatase inhibitors cocktail inhibitor (Calbiochem). The brain lysates were homogenized with pellet pestle motor (Kontes) and were kept on ice for 15 minutes to further enhance the lysis before centrifuging at 16,100 x g for 15 min at 4 °C. The lysate supernatant was then ready and used immediately for the quantification of total protein levels. Supernatants were collected and assayed for total protein using the Bradford method (Biorad, Richmond, California). Samples were stored at -80 °C until needed.

2.5.2. **Cell lysates**

Protein extracts were prepared from N2a and HEK293 cells. Briefly, the culture media was removed and the cells were washed thrice in ice cold sterile PBS. The culture plates were drained thoroughly before adding TNE lysis buffer (10 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP40) supplemented with protease and phosphatase inhibitors (Calbiochem). The cell lysates were collected using a sterile scraper and transferred into 1.5 ml Eppendorf tubes kept on ice. The tubes were incubated on ice for 30 min at 4 °C before centrifuging at 16,100 g for 10 min at 4 °C in order to remove insoluble cellular components. The lysate supernatant was then transferred to a new tube and analysed immediately for protein quantification.
2.6. Subcellular membrane-cytosol fractionation of brain samples

Brain tissue or cells were lysed in 200 µl of KHEM buffer (50 mM KCl, 50 mM HEPES, KOH Ph7.2, 10 mM EGTA, 1.92 mM MgCl₂) supplemented with protease and phosphatase inhibitors (Calbiochem) and sat on ice for 30 min to enhance lysis. Lysates were spun down for 10 minutes at 1000 g at 4 °C (the low speed pellet precipitates the cell debris and nuclei) and the lysate supernatant was transferred to a new 1.5ml eppendorf tube. The supernatant was then centrifuged at 100000 g for 1h at 4°C in a Beckman ultracentrifuge. The supernatant was saved as the cytosol fraction. The pellet (high speed pellet) was resuspended in KHEM buffer +1% triton and 150mM NaCl and incubated for 30 minutes on ice with occasional agitation before the second centrifugation for 100000 g for 1 h at 4 °C. The supernatant was saved as the membrane fraction. To assess the purity of the fractionation, membrane and cytosolic, membrane and nuclear fractions were confirmed by immunoblotting using anti-GAPDH as cytoplasmic marker and anti-GPR40 as membrane marker.

2.7. Determination of protein concentration

The protein concentration of the brain lysates was determined according to the Bradford dye-binding method (Bradford, 1976) using dye reagent from Bio-Rad (Hampstead, U.K.). The Bradford assay is a colorimetric assay based on the colour change of Coomassie Blue G-250 when it binds to various concentrations of protein. Briefly, Bradford reagent was prepared by diluting 1 part of concentrated Bio-Rad dye reagent with 4 parts of sterile water. A range of bovine serum albumin (BSA) concentrations (1-36 µg/µl) were prepared as protein standards. The protein samples were assayed in duplicates in 96-well microtitre plates. The 96-well plate was then analysed at 595 nm using a SpectraMax M5 (Molecular Device, USA). A standard curve of absorbance against BSA concentration was plotted using least squared regression analysis to provide a relative measurement of protein concentration. The concentration of protein samples was then adjusted to account for any dilution factor.
2.8. Protein analysis

2.8.1. SDS-PAGE

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis was carried out to resolve proteins by their molecular weight and charge. Briefly, equal amounts of protein samples (50 µg) were denatured and reduced in 5x SDS-PAGE sample buffer Laemmli (10 % SDS, 300 mM Tris-Cl; pH 6.8, 0.05 % bromophenol blue, 50 % glycerol, 10% β-mercaptoethanol) followed by boiling at 100 °C for 10 min. After brief centrifugation, protein samples were resolved on precast polyacrylamide gels (4-12 % NuPAGE® Novex Bis-Tris gel, Invitrogen) using Invitrogen X-cell apparatus and NuPage® Novex gel system immersed in MOPS SDS running buffer according to nature of the samples and different protein separation range. Pre-stained precision plus all blue standards protein marker (Bio-Rad, USA) was loaded to the first well of the gel to be used as a molecular weight market while the protein samples were loaded to the subsequent wells. Gels were subjected to electrophoresis at 40 mM for 1.5 h to allow for adequate separation.

2.8.2. Western immunoblotting

For Western blotting, SDS-PAGE gels were electrotransferred onto nitrocellulose membranes (0.45 µm pore, Immobilon-P Membrane, PVDF, Millipore) using Mini Trans-Blot® cell (BioRad) in NuPAGE transfer buffer containing 20 % methanol. Proteins were transferred for 2 h at 150 mA at 4 °C. Transfer efficacy was established through visualisation of the pre-stained molecular weight markers onto the nitrocellulose membrane or Ponceau staining. Following transfer the membrane was then blocked in 5 % (w/v) non-fat dry milk (BioRad) or PhosphoBLOCKER blocking reagent (Cell Biolabs, Inc.) in 1xTBST (20 mM Tris-Cl; pH 7.6, 150 mM NaCl, 0.1 % Tween 20) for 1 h at room temperature under agitating conditions to block non-specific antibodies binding to the membrane. Membranes were then probed with specific primary antibodies (Table 2.2) diluted in 1 % milk/TBST solution and incubated overnight at 4 °C under agitation. The membranes were washed 3 times for 10 mins each in 1xTBST before adding appropriate horseradish peroxidise (HRP) conjugated anti-
immunoglobulin G (IgG) secondary antibody (Santacruz) diluted 1:2000 in fresh prepared 1% milk/TBST solution for 2h at room temperature (RT) (details of antibodies and dilutions given in Table 2.2). After secondary antibody incubation, membranes were washed 3 times for 10 minutes with TBST and detected by enhance chemiluminescence (ECL) Western Blotting Substrate (GE Healthcare). Membranes were incubated in ECL substrate for 1 minute before transferring to a light-sensitive cassette. Chemiluminescent images of immunodetected bands were recorded on blue-light sensitive autoradiography X-ray films (Blue Devil Film, Genesee Scientific, Inc.) which were then developed using the Kodak® X-Omat Model 2000 processor. Immunoblots intensities were quantitatively and analysed using ImageJ (http://imagej.nih.gov/ij/).

Densities of the protein bands were measured by imageJ software and the statistical analysis was done by graphpad prism 7. Results are shown as means ± standard error of mean (SEM). The differences between the means were evaluated by one-way ANOVA followed by Dunett’s post hoc for comparison between the control and tested groups, and the tukey-kramper’s post hoc for comparison between the groups. Differences at p< 0.05 were considered significant.
Table 2.2 List of the primary antibodies used in this study. Application key: WB=Western blot; IP=Immunoprecipitation. The PDE4A5 specific antibody that was produced in house has been described previously (Huston et al., 2000) as a specific pan-PDE4A antiserum.

<table>
<thead>
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<th>Antibody</th>
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<th>Catalogue #</th>
<th>Host</th>
<th>Dilution</th>
<th>Applications</th>
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<td>1/4000</td>
<td>WB/IP</td>
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2.9. Ponceau Staining

Ponceau staining is a reversible way of visualising proteins on a nitrocellulose membrane after their transfer. Membranes are incubated in Ponceau solution (Pierce) for minimum 30 minutes with gentle agitation. Blots were either scanned or washed in dH₂O until staining was removed and then used for Western blotting.
2.10. PDE4 activity assays

The PDE4 activity in the brain homogenates was assayed by a modification of the two-step radio assay procedure of Thompson and Appleman (1971) and Rutten et al. (1973) and has been described previously by Marchmont and Houslay (1980). The samples were lysated in KHEM buffer (50 mM KCl, 50 mM HEPES pH 7.2, 10 mM EGTA, 1.9 mM MgCl2) supplemented with protease (COMPLETE EDTA-free, Roche) and phosphatase (PhosSTOP, Roche) inhibitor cocktail tablets. Pilot assays were carried out to verify PDE4 activity and ensure activity fell within the linear range of 6000-16000 counts. Each sample was done in triplicate and was incubated with and without the phosphodiesterase inhibitor rolipram. Rolipram was dissolved in 100% DMSO as a 10 mM stock solution and diluted in 20 mM Tris/HCl, 10 mM MgCl2 buffer (final pH 7.4) to a final concentration of 10µM. The difference between the two different measurements represents the specific PDE4 activity in each sample.

In the first step, 40 µl of the appropriate amount of µgr of brain lysate dissolved in KHEM buffer and supplemented with protease and phosphatase inhibitors was added to a 100 µl of a reaction mixture. That was further supplemented with 20 mM Tris, pH 7.4 to the final volume of 50µl, 10µl of either diluted rolipram or DMSO and 50µl of 20 mM Tris, pH 7.4 with 5 mM MgCl2 0.1 µCi tritiated cyclic nucleotide and 1µM cyclic AMP. For every 1 ml of 20 mM Tris, pH 7.4, 5 mM MgCl2 that was used, 3µl of the tritiated cAMP and 2 µl of the non-tritiated cAMP were added. The reaction mixture was vortexed and incubated at 30 °C for 10 minutes before termination of the protein activity by boiling for 2 min. When the protein concentration of the samples was too low, 20 minutes of the incubation was performed, but then the final counts for the analysis of the PDE4 activity were divided by two. During this step, the PDEs in every sample are hydrolyzing the 8-[3H]-labelled cAMP substrate to [3H]-5’-AMP. In the second step, after the reaction mixture had cooled to 4 °C for 15 minutes, 25 ul of Crotalus Atrox venom (1 µgr/µl) was added, and incubation of this mixture was carried out for 10 min at 30 °C to attain complete conversion of 5’-AMP to adenosine. After this stage, 0.4ml of 1:1:1 (v/v/v) slurry of Dowex 1X8 200-400 MESH CI resin, ethanol and water were added to the reaction mixture, vortexed and then incubated on ice for at least 15 minutes. Anion exchange resin binds negatively charged,
unhydrolysed cAMP, separating it from the adenosine. This mixture was vortex-mixed several times over a 15 min period before being centrifuged at 13000 g in a table microcentrifuge for 3 min at 4 °C to sediment the resin thoroughly. Samples (150 ul) Dowex/water/ethanol (1:1:1, by vol.) were used for the determination of activities. They were mixed with 1ml Opti Flow SAFE 1 scintillant and counted on a Wallac 1409 Liquid Scintillation Counter. Corrections were made for the cyclic \[^{3}\text{H}]\text{AMP not removed by the resin by using a blank that consists of everything except protein sample. The amount of [3H]-adenosine is then calculated by scintillation counting, to determine the rate of cAMP hydrolysis.}

2.11. Protein-protein interaction assays

2.11.1. In vitro co-immunoprecipitation assay

*In vitro* co-immunoprecipitation (co-IP) was performed for the confirmation of protein interactions. Cells either express proteins of interest, or are transfected with the plasmids that produce the proteins of interest. Media of cells were aspirated gently and cells were washed 3 times with ice cold PBS. Cells were scraped in the lysis in TNE buffer (10mm Tris, pH8.0, 150Mm NaCl, 1mm EDTA PH8.0, 1% NP40) supplemented with protease (Chymotrypsin 1.5 µg/ml, Thermolysin 0.8 µg/ml, Papain 1 mg/ml, Pronase 1.5 µg/ml, Pancreatic extract 1.5 µg/ml, Trypsin 0.002 µg/ml, Roche) and phosphatase (Calf alkaline phosphatase 140u/10ml, potato acidic phosphatase 2u/10ml, human acidic phosphatase 640u/10ml, rabbit PP1 200u/10ml, human PP2A 500u/10ml, human PTP 500u/10ml) inhibitors and were collected in an eppendorf tube. Tubes were incubated on ice for 30 minutes before being centrifuged for 15 minutes at maximum speed at 4 °C. Next the supernatant was transferred to a new tube. Protein concentration was determined using BioRad as it has been described previously. Next, all the samples were normalized to have the same total amount of protein in the same volume. The protein lysate was precleared with Protein A Plus agarose (Thermo Scientific) for 2 hours with rotation at 4 °C. After preclearing, the tubes were spun down at 1000 g for 1 minute at 4 °C and the supernatant was transferred to a new tube. 2-3 µgr/ml of the appropriate antibody was used to immunoprecipitate (IP) endogenous and overexpression of
the proteins of our interest as well as their binding partners (details of antibodies given in Table 2.2) and were incubated overnight rotating. Negative controls using isotype-matched IgG (Santa Cruz Biotechnology) from the same species as the antibodies were included to screen for non-specific binding. The resulting immunocomplexes were captured the next day incubating for 2 hours using Protein A beads (Thermo scientific) for 2 hours rotating. The immunocomplexes were then collected by centrifugation at 1000 g for 2 min and washed five times with TNE lysis buffer supplemented with protease and phosphatase inhibitors. Bound proteins were then eluted in Laemmli (10% SDS, 300 mM Tris-Cl; pH 6.8, 0.05% bromophenol blue, 50% glycerol, 10% β-mercaptoethanol), then boiled at 100 °C for 10 min and subjected to SDS-PAGE. Western immuno-blotting was performed using various antisera as described previously.

2.11.2. Co-immunoprecipitation assay of mouse brain lysates

In vivo co-immunoprecipitation (co-IP) was performed for the confirmation of protein interactions in mice. Mice were fasted for 7 hours during the day and then food was added for 2 hours. Mice were sacrificed and the hypothalamic area was isolated and lysed in TNE buffer (10mm Tris, pH 8.0, 150mM NaCl, 1mm EDTA pH 8.0, 1%NP40) supplemented with protease and phosphatase inhibitors. The lysates were processed as it has been described in the section above.

2.12. RNA Expression Analysis

Real-time PCR has been widely used to determine relative gene expression. This detection method is based on the changes of fluorescence accumulation during thermocycling which can be reflected on a sigmoidal amplification plot and give a quantitative result of the amplified product (Kubista et al., 2006). Variability in qPCR can be influenced by steps upstream which consist of template preparation and reverse transcription.
2.12.1. RNA extraction

Total RNA was extracted using the QIAGEN lipid tissue kit with QIAZOL which contained phenol and guanidine thiocyanate in a procedure based on the method of Chomczynski and Sacchi (1987). Brain homogenates where first lysed in 600 ul of QIAZOL with a pellet pestle motor (Kontes) and tubes were incubated for 5 min at room temperature. RNA was separated from DNA and proteins by the addition of 200 ul of chloroform. After vigorous vortexing for 15 sec, the tubes were incubated at room temperature for 2-3 min. The aqueous phase, which contains the RNA, was then separated by centrifugation at 12,000 x g for 10 minutes at 4 °C. The aqueous phase was transferred to a new tube, and the RNA was precipitated by adding an equal volume of 70% ethanol and mixing thoroughly. The samples were then transferred to an RNeasy mini spin column and centrifuged for 15 seconds at 8,000 x g at room temperature. The flow-through was discarded. Next, the mini spin column was washed with 700 µl of the RW1 buffer and the flow-through was discarded. The RNeasy spin column was further washed twice with 500ul of RPE buffer and the flow-through was discarded. After the washes, the RNeasy spin column was placed in a new 1.5 ml collection tube (supplied) and 30 µl of the RNase-free water were added directly to the spin column membrane. The spin column was then incubated at room temperature for 5 minutes and the RNA was eluted by centrifugation for 5 min at 8000 x g (10,000 rpm).

2.12.2. Gene expression profiling by microarray analysis

Microarray analysis was performed on hypothalamic areas of mice fed normal diet and either 4 or 8 weeks on a high-fat diet. Hypothalamic area was dissected using the brain slicer matrix (Zivic instruments). Total RNA was isolated with RNeasy Mini kit/RNeasy Lipid tissue mini kit (QIAGEN) according to the manufacturer’s instruction. DNase I treatment of the RNA fraction was carried out using an RNase free DNase set (QIAGEN) as a precaution against genomic DNA contamination of the RNA. Probes were prepared using NuGEN Ovation Pico WTA V2 kit and NuGEN Encore Biotin Module, and hybridized to Rat and Mouse Gene 1.0 ST GeneChip arrays (Affymetrix). Arrays were scanned using an Affymetrix GCS3000 scanner and Affymetrix Command Console software, and data were
normalized using the RMA algorithm in Affymetrix Expression Console. Microarrays were normalized for array-specific effects using Affymetrix's "Robust Multi-Array" (RMA) normalization. Normalized array values were reported on a log$_2$ scale. For statistical analyses, we removed all array probe sets in which no experimental groups had an average of log$_2$ intensity greater than 3.0. This is a standard cutoff, below which expression is indistinguishable from background noise. Linear models were fit for each gene using the Bioconductor "limma" package in R (Gentleman et al., 2004). Moderated t-statistics, fold-change and the associated P-values were calculated for each gene. To account for the fact that thousands of genes were tested, we reported false discovery rate (FDR)-adjusted values, calculated using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). FDR values indicate the expected fraction of falsely declared differentially expressed ("DE") genes among the total set of declared DE genes (i.e., FDR = 0.15 would indicate that ~15% of the declared DE genes were expected to be due to experimental noise instead of actual differential expression).

2.12.3. Reverse transcription of PCR

3 µl of RNA was reverse-transcribed to first strand complementary DNA (cDNA) using GeneAmp RNA PCR core kit (Applied Biosystems) according to manufacturer’s instructions. A reaction mixture containing 5 mM MgCl$_2$ final concentration, 1 mM each dNTP), 1 unit of RNase Block Ribonuclease Inhibitor and 2.5 units of reverse transcriptase (RT) MULV was added to the random hexamer primer mixture and incubated at 42 °C for 20 minutes. The random hexamer primers bind to random places on the RNA so that the reverse transcriptase can polymerase. The reaction was then terminated by heating at 99 °C for 10 min and subsequently chilled on ice for 10 min to deactivate the enzyme. The cDNA was stored at -80 °C until use.

2.12.4. Quantitative real-time PCR with SYBR green

Specific real time PCR primer sets (Eurofins MWG operon) for each gene were from the primer bank or designed assisted by the Primer 3 software (http://frodo.wi.mit.edu/primer3/). Table 2.3 shows the sequences of the
different real time PCR primers that were used. The primers were designed to span an exon-intron-exon boundary to exclude amplification of genomic DNA. GAPDH was used as an internal control for normalising relative expression levels in the different samples. Real-time PCR was performed from reverse transcribed cDNA samples using the SYBR Green PCR master mix (Applied Biosystems, Invitrogen) following the manufacturer’s instructions. Briefly, 2 µl of cDNA were added to a 96-well MicroAmp® Fast Optical Reaction Plate (applied Biosystems) with 12.5 µl of SYBR Green and 1 µl each of the 10 µM primer pair. Thermal cycling and fluorescent monitoring were performed using StepOne Plus Real-time PCR system (Applied Biosystems). Each PCR amplification was run in duplicates using the following conditions: initial denaturation at 95 °C for 10 min, followed by a total of 40 cycles (15 sec at 95 °C for denaturation, 1 min at 60 °C for extension). Fluorescence data were collected during the extension step of each cycle. Negative controls using water instead of cDNA as template were also included in all runs.
Table 2.3. List of real time PCR primers. List of the pcr primers used for real time PCR analysis

<table>
<thead>
<tr>
<th>Primer name</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPDE4D_Fw</td>
<td>GTCCCATGTGTGACAAGCAC</td>
</tr>
<tr>
<td>mPDE4D_Rv</td>
<td>TCAGTGTCGACTCGCCTATC</td>
</tr>
<tr>
<td>mPDE4A_Fw</td>
<td>CGAGCAGCTACAGTGTTGGGAA</td>
</tr>
<tr>
<td>mPDE4A_Rv</td>
<td>AAGGAATCGACGACAGGGGTCT</td>
</tr>
<tr>
<td>mPDE4B_Fw</td>
<td>GTCCAGTGTGTTGTTGATT</td>
</tr>
<tr>
<td>mPDE4B_Rv</td>
<td>ACACAGGGAATGGAATCGAGA</td>
</tr>
<tr>
<td>mGAPDH_Rv</td>
<td>GGCTGACCCCATTTGATGT</td>
</tr>
<tr>
<td>mGAPDH_Fw</td>
<td>CAAGGCAGAGAATGGAAG</td>
</tr>
<tr>
<td>mPDE4A5_Fw</td>
<td>TCGCCGACGGCCCATAGA</td>
</tr>
<tr>
<td>mPDE4A5_Rv</td>
<td>GAGCAGGGAACAAATGCGA</td>
</tr>
<tr>
<td>mTNF-α_Fw</td>
<td>CCCTCACACTCAGATCATCTCT</td>
</tr>
<tr>
<td>mTNF-α_Rv</td>
<td>GCTACCAGCTGGCTACAG</td>
</tr>
<tr>
<td>mIL-1β_Fw</td>
<td>GCAACTGTTCCTGAACTCAACT</td>
</tr>
<tr>
<td>mIL-1β_Rv</td>
<td>ATCTTTGCGTCCGTAACACT</td>
</tr>
<tr>
<td>mIFN-γ_Fw</td>
<td>CAGAGCCAGATTATCTCTTCTACCTACCTACCTACAG</td>
</tr>
<tr>
<td>mIFN-γ_Rv</td>
<td>CTCTTCTCGCCCTGTTGCTGATGA</td>
</tr>
<tr>
<td>mBDNF_Fw</td>
<td>AGGCACGGGTGAATCGCAATG</td>
</tr>
<tr>
<td>mBDNF_Rv</td>
<td>AAGGCCAGACATACGATT</td>
</tr>
<tr>
<td>mGPR41_Fw</td>
<td>CTGTATCGACCCGCTTTTT</td>
</tr>
<tr>
<td>mGPR41_Rv</td>
<td>GCTGAGTCAGGCGACACAGA</td>
</tr>
<tr>
<td>mGPR120_Fw</td>
<td>TTCTATGGGTTACTGCGGC</td>
</tr>
<tr>
<td>mGPR120_Rv</td>
<td>GATTTCCTATATGGCGGG</td>
</tr>
<tr>
<td>mGPR40_Fw</td>
<td>AATGCCTGCAATGCGCTA</td>
</tr>
<tr>
<td>mGPR40_Rv</td>
<td>ATCTCCTGTCACACATATTG</td>
</tr>
</tbody>
</table>
2.12.5. Quantitative real-time PCR analysis

Relative changes in gene expression of the target genes in different samples were quantified using the comparative Ct method (ΔΔCt) as described by Livak and Schmittgen (2001). The objective of this method is to compare the PCR signal of a target gene in a treatment group to the untreated control after normalizing to an endogenous reference gene. Firstly, the point at which the PCR was detected above a set threshold in exponential phase, termed threshold cycle (Ct), was obtained from the real-time PCR instrumentation. Ct is determined from a log-linear curve where PCR signal is plotted against the cycle number. The Ct values of each sample were then imported into Microsoft Excel and the average Ct of duplicate samples was calculated. The amount detected at a certain cycle number is directly related to the initial amount of target in the sample. Hence, to determine the quantity of gene-specific transcripts present in cDNA, Ct values for each treatment had to be normalized first to obtain ΔCt. This was accomplished by subtracting the mean Ct value of endogenous reference gene 18s rRNA of each group from the corresponding mean Ct value of gene of interest (GOI) accordingly (ΔCt = CtGOI - ΔCt18s rRNA). The concentration of gene-specific mRNA in treated cells relative to untreated cells was then normalized through subtraction again to obtain ΔΔCt values (ΔΔCt = ΔCt of treated cells - ΔCt of untreated cells). Finally, the relative expression which is often termed as RQ value was determined by raising 2 to the power of the negative value of ΔΔCt (2-ΔΔCt) for each sample (Amount of target = 2-ΔΔCt). Alteration in mRNA expression of target genes was defined as fold difference in the expression level in cells after the treatment relative to that before treatment. Samples with poor technical replicate values were excluded from analysis.
2.13. **Fatty acid analysis by gas chromatography–mass spectrometry.**

The total concentrations of palmitic acid (16:0), stearic acid (18:0), myristic acid (14:0), behenic acid (22:0), arachidic acid (20:0), gondoic (20:1), oleic (18:1) and linoleic (18:2) were determined from tissues using gas chromatography–mass spectrometry (Brunengraber et al., 2003). A known quantity of tissue was hydrolyzed and extracted after adding a known amount of heptadecanoic acid (17:0). Fatty acids were analyzed as their trimethylsilyl derivatives under electron impact ionization mode using an Agilent 5973N-MSD equipped with an Agilent 6890 GC system and a DB17-MS capillary column (30 m × 0.25-mm internal diameter × 0.25-µm film thickness).

2.14. **cAMP measurement of mouse brain lysates**

Measurements of cAMP *in vivo* were performed using the CatchPoint Cyclic-AMP fluorescent assay kit (Molecular Device, CA). Briefly, whole hypothalamic brain samples were lysed in lysis buffer provided by the manufacturer and left on ice for 15 minutes. Lysates were spun down for 10 minutes at 1000 g at 4°C (low speed pellet, cell debris and nuclei) and the supernatant was transferred in a new 1.5ml eppendorf tube and was used for the assay. The kit was performed according to manufacturer’s instructions.

2.15. **FRET imaging**

Förster Resonance Energy Transfer (FRET) is a well established technique to study protein-protein interactions as well as second messenger signalling. This phenomenon was first described by Theodor Förster (Förster, 1948) and describes the interactions between the electron excitation states of two fluorophores. Excitation energy is transferred from the donor fluorophore to the acceptor fluorophore without emission of a photon, a process which is non-radiative. The Förster distance, when FRET efficiency is 50% ($R_0$), correlates with the distance between the donor and acceptor fluorophores but an essential requirement is that the distance ($r$) between the fluorophores must be less than 10nm for energy transfer to occur (Kalab and Soderholm, 2010). FRET imaging
experiments were performed 24-48 h after transfection with the PKARI sensor on mouse neuroblastoma (N2a) cell line that were seeded onto glass cover slips. Cells were maintained at room temperature in DPBS (Invitrogen, UK), with added CaCl$_2$ and MgCl$_2$, and imaged on an inverted microscope (Olympus IX71) with a PlanApoN, 60X, NA 1.42 oil, 0.17/FN 26.5, objective (Japan). The microscope was equipped with a CCD camera (cool SNAP HQ monochrome, Photometrics), and a beam-splitter optical device (Dual-channel simultaneous-imaging system, DV$^2$ mag biosystem (ET-04-EM)). Imaging acquisition and analysis software used was Meta imaging series 7.1, Metafluor, and processed using ImageJ (http://rsb.info.nih.gov.ucsf.idm.oclc.org/ij/). FRET changes were measured as changes in the background-subtracted 480/545-nm fluorescence emission intensity on excitation at 430 nm and expressed as either $R/R_0$, where $R$ is the ratio at time $t$ and $R_0$ is the ratio at time $= 0$ s, or $\Delta R/R_0$, where $\Delta R = R - R_0$. Values are expressed as the mean ± SEM. Cells were pretreated with 100uM of either palmitic or oleic before 5uM of forskolin treatment. At the end of every experiment, saturated doses of forskolin (25uM) or IBMX (100uM) were used to check for the responsiveness of the cells.

2.16. Glucose and insulin tolerance tests

To determine the relative sensitivity or resistance to glucose or insulin, the glucose and insulin tolerance tests were performed on PDE4A$^{+/+}$ and PDE4A$^{-/-}$ mice by measuring blood glucose following either a glucose load or insulin dose. Mice that were fasted for 6 hours during the day received an intraperitoneal injection of either glucose (1-2 milligram/kilogram body weight; 0.01 milliliter/kilogram body weight in sterile PBS) or bovine insulin (0.4U/kilogram body weight). Blood samples (one drop) were obtained by tail bleeding at 10, 30, 60, 90 and 120 minutes after initial injection to measure blood glucose levels using an OneTouch Ultra2 sugar glucometer (Lifescan, Inc. Milpitas, CA).
2.17. Plasmid DNA

For the mammalian expression of wild-type PDE4A5-vsV and arrestin-2, constructs were provided by Professor George Baillie. Mouse GPR40 construct was obtained from Origene. All plasmid work was carried out in sterile conditions and all buffers made in-house and were autoclaved prior to use.

2.17.1. Transformation

For mammalian transfection purposes One Shot® DH5a (Invitrogen, USA) chemically competent E. coli cells were used for plasmid production. Chemically competent cells were stored at -80 °C and thawed on ice immediately prior to use. Approximately 1-10 ng of appropriate plasmid DNA was added to 50 ul of competent cell and mixed gently. Cells were then incubated on ice for 30 minutes then heat-shocked for 30 seconds at 42 °C then returned to ice for 2 minutes before the addition of 200 µl of super optimal broth with glucose (SOC media) (Invitrogen, USA) to the cells. Cells were then incubated at 37 °C for 1 hour at 300 rpm to allow for recovery of the cells. The transformation mix was then spread on 100 mm pre-warmed agar petri dishes made with sterile Luria-Bertani medium (LB) (1 % tryptone (w/v), 0.5 % yeast extract (w/v), 170mM NaCl) supplemented with appropriate antibiotic either kanamycin (50 µg/ml) or ampicillin (100 µg/ml). Plates were incubated upside down at 37 °C overnight. The growth of bacterial colonies indicated successful cell transformation.

2.17.2. Amplification & isolation of the plasmid DNA

In order to amplify the plasmid DNA, either single colonies were picked from the plate or from frozen glycerol stocks under sterile conditions and grown during the day in 5 ml LB media containing either kanamycin or ampicillin in an orbital shaker at 37 °C at 220rpm. The 5 ml bacterial culture was added in a 200 ml flask supplemented with the appropriate antibiotic and incubated a further 12-16 h. The next day, the culture was pelleted and plasmid DNA was purified in accordance with Midi kit (Qiagen), in accordance with manufacturer’s instructions. QIAprep Miniprep Kit (Qiagen) was used to isolate smaller amounts of plasmid DNA. The purified DNA was then eluted with sterile H2O and stored at
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-20 °C. The resulting plasmid DNA was sequenced by the MCLAB services (www.http://www.mclab.com) in order to verify the correct sequence.

2.17.3. Quantification

The concentration of the purified plasmid DNA was determined using a Nanodrop 3300 spectrophotometer (Thermo-Scientific). The ratio of absorbance at 260 nm and 280 nm determines the purity of the DNA where a value of 1.8 is indicative of highly purified DNA. The DNA concentration was calculated using the Beer-Lambert law, where an A260 reading of 1.0 optical density (OD) unit is equivalent to 50 µg/ml double-stranded DNA (Sambrook et al., 1989); which is done automatically by the Nanodrop software.

2.17.4. Plasmid Storage

For long-term plasmid storage, 750 ul of overnight culture was added to 250 µl of 70 % glycerol in a sterile cryovial, snap-frozen on dry ice then stored at -80 °C until required. For amplification of the plasmid DNA of the glycerol stocks, cryovials were placed on dry-ice to prevent thawing and frozen stocks and were scraped using a sterile pipette tip and transferred to LB containing appropriate antibiotic.

2.17.5. Cloning of the PDE4A5-VSV into the AAV vector

The adeno-associated vector (AAV) expressing GFP under the synapsin promoter was provided by the vector core of the University of Pennsylvania. Figure 2.6 shows a schematic of the AAV of the construct.
At the same vector the substitution of GFP with PDE4A5-vsv was performed in order to achieve overexpression of PDE4A5 under the synapsin promoter. The GFP was excised using EcoRI and XhoI (England Biolabs). Then the PDE4A5-vsv was amplified with a set of primers containing the restriction sequences of EcoRI and XhoI. The PCR fragment containing the EcoRI-PDE4A5-vsv-XhoI was ligated with the digested vector using the rapid DNA ligation kit (Roche #11635379001). Briefly, the digested vector and the insert were mixed together in a ratio of 1:3 and they were further diluted in the dilution buffer in total volume of 10 µl. The 10 µl of the ligation mix was incubated with 1 µl of 5 units of ligase at RT for 30 minutes. Next, the ligation product was used to transform the stbl2 bacteria (Invitrogen) that is suitable for unstable DNA. The cloning was confirmed by sequencing the vector. Figure 2.7 represents a map of the construct that was produced.
Figure 2.7. Map of the adeno-associated virus used to express the rat PDE4A5-wt under the synapsin promoter. Construct was obtained by the Gene therapy vector core at the University of Pennsylvania and cloning was performed in order to add the PDE4A5-vsv.
2.18. *In vivo* delivery of AAV vector expressing PDE4A5 and GFP

To investigate the role of PDE4A5 in depression, adeno-associated virus (AAV) encoding the PDE4A5-wt rat isoform tagged with vsv and under the synapsin promoter was generated to induce overexpression of the PDE4A5 in neurons ([Fig. 2.2](#)). Synapsin promoter has been used widely to drive neuronal overexpression. The pde4a5 was tagged with vsv so that we could distinguish between the native and the externally overexpressed pde4a5. As a control was used the same construct overexpressing GFP under the synapsin promoter ([Fig. 2.1](#)). The production of the virus was carried out at the core of gene therapy at the University of Pennsylvania. As anesthesia for the surgeries, we used avertin as it has been shown that there is a possible interaction between xylazine/ketamine anesthesia and pde4 (Robichaud et al., 2002). AAV vector expressing either PDE4A5-WT-vsv or AAV-Egfp were injected bilaterally into the thalamus using the following coordinates measured from the bregma AP -1.5mm, ML ±0.4mm, DV -5.8mm from dura (Paxinos and Watson 1998). One microliter of the AAV-PDE4A5-vsv (4.99x10^{13} infectious particles/ml) was infused over a 5 minute period. AAV control GFP (5.5x10^{13} infectious particles/ml) was used as a control. Following injection, the injector was kept in place for additional 5 minutes to allow for diffusion and prevention of backflow through the needle track before the injector was withdrawn. After surgery, the mice were provided with high fat diet for 3 weeks. Upon the completion of the three weeks, behavioral assays were performed to assess the depression phenotype. Upon the completion of all the behavioral assays, mice rest for 2 days before rapid decapitation. The hypothalamic area was extracted and snap frozen in liquid nitrogen. The injection site was confirmed by visualizing GFP fluorescence suing a fluorescent microscopy.
2.19. Mammalian cell culture

All cell culture procedures were carried out in a Class II hoods using standard aseptic techniques and sterile equipment. All culture reagents such as media were supplied by Invitrogen while the tissue culture flasks, dishes and pipettes were supplied by Corning. All cultures were examined regularly under a phase contrast inverted microscope (Leitz Diavert, Germany or the Nikon Eclipse TS100) to confirm the healthy status of the cells and monitor for contamination. N2a and HEK293T cell lines were maintained at in 10 cm$^2$ plates at 37 °C in a humidified atmosphere of 5% CO$_2$ and 95% air. Culture media was routinely replaced every 3-4 days and cells upon reaching 80% confluency were passaged. To passage, growth medium was removed and the cells were gently washed in sterile pre-warmed phosphate buffer saline (PBS) to remove traces of serum. The PBS was then discarded and the cells were treated with 0.5 ml of trypsin-EDTA solution per 10 cm$^2$ plate of cells to dissociate cells in the monolayer. 8 ml of growth medium was then added to inactivate the trypsin-EDTA solution and cells were collected by centrifugation at 12,000 rpm for 3 min at room temperature. The supernatant was discarded and the cell pellet was resuspended in fresh growth media and added to fresh culture flasks at the required dilution. The volume of PBS, trypsin-EDTA and growth media were adjusted according to the size of the culture flask. When cells were harvested for long term storage, the cells were resuspended in 10% dimethyl sulfoxide (DMSO) containing 90% FBS and stored for 3 days in -80 °C before being transferred in liquid nitrogen. For the freeze down, cells were placed in a container that reduces the temperature by 1°C/minute.

N2a cell line culture: The mouse neuroblastoma cell line (N2a) were maintained in growth media containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine, 1% (v/v) penicillin-streptomycin and 1% (v/v) non-essential amino acid (NEAA).

HEK293 cells: The human embryonic kidney 293 (HEK293) cells were maintained in growth media containing DMEM supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine, 1% (v/v) penicillin-streptomycin.
2.19.1. Transfection of cells with plasmid DNA

DNA plasmid constructs used for transfection included PDE4A5-vsv, GPR40, arrestin-2 in pcDNA3 vectors. For transient expression, HEK293 or N2a cells were plated at a density of $2 \times 10^6$ cells per 100 mm dish. Transfections were performed at 50-60% confluence with 5 µg of total circular plasmid DNA using the linear MW~25000 polyethylenimine (Polysciences Inc). For the HEK293 cells 2ugr of PDE4A5-vsv, 2ugr of GPR40 and 1ugr of arrestin-2 were used per 100 mm plate. For the N2a cell line that already expresses GPR40, 2.5ugr of PDE4A5-vsv and 2.5ugr of arrestin-2 were used per 10 mm plate.

Briefly, the plasmid DNA was incubated for 5 minutes with 250 µl of 150 mM sterile NaCl. In parallel, 30 µl of 1 µg/µl of polyethylenimine (PEI) was incubated with 250 µl of 150 mM sterile NaCl. After the 5 minutes, the two tubes containing the DNA complexes and the PEI were mixed together for 30 minutes before adding the mixture to the cells. The cell media was changed 15 minutes before the addition of the DNA with PEI. After 24 h transfection, the medium was replaced with fresh pre-warmed culture medium and was further incubated for 24 h prior to experiments. This assay was scaled down for smaller culture dishes. For microscopic FRET experiments, cells were plated out onto poly(L-lysine)-treated glass cover slips at approximate 40% confluence.

2.19.2. In vitro fatty acid treatment

Palmitic, oleic and myristic acid were purchased from Nu-check and were used in this study. The treatment of cells with the different fatty acids was done by reducing the serum before the actual experiment. The day before the in vitro experiment, cells were washed twice with pre-warmed DMEM and a medium with 0.3% serum was added to the cells. The next day in the morning the cells were washed twice with pre-warmed DMEM and medium was added without any serum for 5 till 8 hours. A stock of 100 mM solution was made for the different fatty acids and prepared fresh every time. For the fatty acid treatments a stock solution was further diluted in pre-warmed DMEM and then it was added on cells.
2.20. Statistical analysis

Data were expressed as mean value ± standard error of the mean (SEM) and an alpha level of 0.05 was used as marker of statistical significance. Statistical significances between two groups of data were determined using unpaired, two-tailed Student’s t-test. Statistical analysis of several groups was carried out either by using one way analysis of variance (ANOVA) with different post-test comparisons against control experiments using GraphPad Prism 7 or mixed model analysis with fixed and random factors using R. A p-value greater than 0.05 was not considered significant (NS), p-value < 0.05 was labelled as (*), p-value < 0.01 was labelled as (**), and p-value < 0.001 was labelled as (***).
Chapter 3

Dietary or genetic obesity induces a depression phenotype in mice
3. Introduction

3.1. Introduction

Obesity is a multicomponent syndrome that results from an imbalance in energy homeostasis. Obesity has been linked with various other diseases, one of which is depression. Many different epidemiological studies have revealed an association between obesity and depression (Zhong et al., 2010; Leonore de Wit and Frans Zitman, 2010; Boutelle et al., 2010; Stunkard et al., 2003; Simon et al., 2010; McElroy et al., 2004; Faith et al., 2002; Luppino et al., 2010; Palinkas et al., 1996). On one hand, being overweight or obese is an indicator for depression later in life and conversely, antidepressant treatment has been linked with body weight gain; whether this increase is due to euphoria or a side effect of the treatment remains unknown. As the prevalence of obesity in our modern societies is increasing dramatically, any potential positive correlation with depression will have detrimental effects for the society. Studies in mice have suggested that diet-induced obesity induces depression (Sharma and Fulton, 2013). However, the mechanisms that link obesity and depression remain unknown. Understanding the link between obesity and depression and whether one of the two can play a causative role for the development of the other is of clinical and social importance.

3.1.1. Obesity models in rodents

Several genetic models of obesity in rodents have been described (Robinson et al., 2000) with five historically naturally occurring mutations (Friedman and Leibel, 1992) that led to major breakthroughs in understanding the mechanism of obesity and metabolic syndrome. The discovery of leptin (Zhang et al., 1994), an adipose tissue hormone and its receptor in the brain (Chen et al., 1996; Lee et al., 1996) was a significant breakthrough as it highlighted the importance of the communication between peripheral tissues and the central nervous system that controls energy homeostasis (Schwartz et al., 2000).
High fat diets (in which 60% of the calories come from fat) are often used to induce obesity and metabolic disorders in rodents that are comparable to human metabolic syndrome (Buettner et al., 2007). Even though the genetically developed obese mouse models have increased our understanding of obesity, consumption of a high fat diet (HFD) is conceived to be a common cause of obesity in humans (Hill and Peters, 1998). Therefore, the animal models of HFD induced obesity better mimic the pathophysiological changes in overweight and obese individuals (Stein and Colditz, 2004; Freire et al., 2005; Moreno and Rodriguez, 2007).

3.1.1.1. *Ob/ob*: the genetic mouse model of obesity

Leptin is an adipocyte-derived hormone that was discovered in the 1950s (Ingalls et al., 1950) when a natural mutation occurred in a mouse strain resulting in an autosomal recessive morbid obesity syndrome called *ob/ob* or obese. Parabiosis experiments in these *ob/ob* or obese mice with normal mice produced profound weight loss in the obese partner, suggesting that the obese gene was encoded by a circulating factor (Coleman, 1973). Positional cloning of the obese gene identified the product as a secreted protein of 17kD later named leptin (Zhang et al., 1994). Leptin enters the brain via a saturable transport mechanism (Banks et al., 1996) and has been described as a pleiotropic hormone as it affects multiple physiological processes such as appetite, body weight, neuroendocrine function and emotional behaviours (Berthoud and Morrison, 2008; Elmquist et al., 2005; Friedman and Halaas, 1998; Lu, 2007; Myers et al., 2009; Licinio et al., 2007; Myers et al., 2008).

Leptin exerts its effects by activating the leptin receptors (Lepr or db) in the brain (Ahima and Flier, 2000; de Luca et al., 2005). There are at least five different leptin receptors (designated Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re) which are derived via alternative splicing of the db gene (Lee et al., 1996) and are widely expressed in different brain regions (Burguera et al., 2000; Elmquist et al., 1998; Smedh et al., 1998; Mercer et al., 1998). Many studies have focused on Lepr-containing neurons within the subdivision of hypothalamus and suggested that the hypothalamic targets are essential for the regulation of the centrally involved action of leptin on food intake, body weight and energy.
homeostasis (Balthasar et al., 2004; Cowley et al., 2001; Dhillon et al., 2006) (Leinninger et al., 2009; Friedman and Halaas, 1998; Schwartz et al., 2000; Cheung et al., 1997). However, even though the effects of leptin on the hypothalamic centres is well described, leptin receptors are widely expressed in numerous extra hypothalamic regions of the brain including the hippocampus, cerebellum, amygdala and brain stem (Elmqquist et al., 1998; Tartaglia et al., 1995; Fei et al., 1997), where leptin’s action remain unclear. A number of recent studies documented the role of leptin in cognition in the hippocampus (Flier, 1998; Harvey et al., 2005; Shanley et al., 2001; Morrison and Berthoud, 2007).

Leptin deficiency results in the onset of obesity as early as 3 weeks of age. The main characteristics of the \textit{ob/ob} mice are increased body weight due to excessive increase of adipose tissue, hyperphagia and hyperdypsia, hyperglycemia, hyperinsulinemia, reduced energy expenditure, dyslipidemia, decreased body temperature and defective thermogenesis (Bray and York, 1979; Campfield, 2000). Elevation of leptin levels induces an anorectic response (Halaas et al., 1997) and it has been shown that leptin administration reduces hyperphagia and obesity in leptin-deficient mice and humans (Campfield et al., 1995; Farooqi et al., 1999; Halaas et al., 1995; Pellemounter et al., 1995).

Although obese humans and rodents usually have high circulating levels of leptin in proportion to their greater body fat mass (Frederich et al., 1995; Maffei et al., 1995), the high concentration of leptin neither reduces food intake nor increases energy expenditure (Schwartz et al., 1996). This paradoxical situation in obesity has been termed “leptin resistance” (Hamann and Matthaei, 1996) and is considered to be a central dogma for obesity in humans and diet-induced obese rodents (Myers et al., 2008). Leptin resistance has been described in both normal and obese animals, and this in turn results in failure to regulate energy expenditure and appetite (Frederich et al., 1995). Thus, the primary physiological role of decrease leptin concentration is to initiate a feeling of starvation and the molecular mechanisms regulating appetite (Ahima et al., 1996). Due to the absence of leptin antagonists, the obese mouse model continues to be an invaluable tool for the study of obesity.
3.1.1.2. Dietary induced obesity models in rodents

Susceptibility of diet induced obesity has been described for the different mouse strains used in laboratory research, with significant variability in the data from different experimental procedures (West et al., 1992). The C57Bl/6J mouse strain is the most commonly used for the study of diet induced obesity in rodents. High-fat diet has been shown to cause the development of obesity and the metabolic syndrome, characterized by hyperglycemia, hyperinsulinemia, and hyperleptinemia, hyperlipidemia, endothelial dysfunction and hypertension, in C57Bl/6J mice (Surwit et al., 1988; Kim et al., 2000; Roberts et al., 2001). The inbred C57Bl/6J strain has a normal weight (~25 g) on standard chow diet, but becomes obese (~42 g) when fed a high-fat diet for longer than 6 weeks (Surwit et al., 1988). The increase of glycogen synthase kinase-3 activity in adipose tissue observed in C57Bl/6J mice is believed to be one of the potential factors that contribute to susceptibility of this mouse strain to diabetes (Eldar-Finkelman et al., 1999). The C57Bl/6J strain is a useful model for human diabetes, as it models the development of obesity and diabetes with high-fat diet that is found in humans (Stein and Colditz, 2004; Freire et al., 2005; Moreno and Rodriguez, 2007).

3.1.2. Models of depression in rodents

Depression is characterized by cognitive symptoms (guilt, ruminations and suicidality), emotional symptoms (anhedonia), homeostatic or neurovegetative (abnormalities in sleep and appetite, weight and energy) and psychomotor agitation or retardation, most of which are difficult to mimic in animal models. None of these characteristics has been proven sufficiently robust or consistent enough to be used for the diagnosis of depression in humans or validate an animal model (Krishnan and Nestler, 2008; Holmes, 2003; Berton et al., 2012). Only a subset of these characteristics such as anhedonia and psychomotor behaviour are relevant to human depression and can be measured in mice (Holmes et al., 2003; Nestler and Hyman, 2010). Therefore, the development of a mouse model that will fully mirror the human neuropsychiatric disorder of depression has proven impossible. However, some behavioural paradigms have been developed to assess the antidepressant effect of different drugs.
Stress is a predisposing risk factor for depression and a variety of experimental models of depression have utilized various stressors to induce depression like behaviours in mice (Bourin et al., 2001; McArthur and Borsini, 2006). Some of these tests rely on the effects of stress responses such as cognitive or attentional impairment and abnormalities in psychomotor activity (Cryan and Mombereau, 2004; Nestler et al., 2002a). Unpredictable mild stress can induce a depression phenotype that is reversed by chronic antidepressant administration (Willner et al., 1987). However, short-term and long-term corticosterone administration after stress results in opposite effects on depression phenotype (Zhao et al., 2009). Psychosocial stress plays an important role in many cases of human depression (Coyne and Downey, 1991; Kessler, 1997) and recently published studies in mice have validated chronic social defeat as an animal model of depression (Malatynska and Knapp, 2005; Tsankova et al., 2006).

The novelty-induced hypophagia (NIH) paradigm provides a promising new model for investigations into the neurobiology underlying the antidepressant response (Dulawa and Hen, 2005). Social isolation is enough to induce a depression phenotype and environmental enrichment showed an antidepressive and anxiolytic effect in animal models of depression and anxiety (Brenes Saenz et al., 2006). Even though unpredictable chronic mild stress has been shown to induce a depression phenotype, that effect depends on the mouse strain (Pothion et al., 2004).

The most common mouse models of depression involve a period to induce anxiety behaviour. The anxiety phenotype has been proven sufficient enough for the development of a depression phenotype. The widely used behavioural tests to induce depression are the forced swim (FST) (Petit-Demouliere et al., 2005; Porsolt et al., 1977a) and tail suspension test (TST) (Cryan et al., 2005; Cryan et al., 2002a; Nestler et al., 2002b). Even though they are not models of depression, they are rapid tests that were developed decades ago for the screening of antidepressant drugs. During these tests, rodents are subjected to an acute, short-duration (minutes) stress and the time during which they respond actively versus passively is measured. A major weakness of these two tests is that they involve short-term stress applied to rodents, which is very
different from human depression in which an underlying genetic vulnerability combines with stochastic and chronic environmental exposures to produce long-lasting behavioural pathology. Despite this, they have proved useful in numerous studies for the screening of antidepressant drugs (Porsolt et al., 1977a; Cryan et al., 2002a).

A second major class of depression-related behavioural tests involves measuring anhedonia, which is the loss of interest in pleasure-giving activities during day-to-day life. The most commonly used test to evaluate anhedonia is the sucrose preference test (SPT), which examines a preference for a sucrose solution over water. Decreased sucrose preference, not resulting from a motor or sensory deficit, is interpreted as a demonstration of anhedonia and thus depression like behaviour (Nestler and Hyman, 2010). The advantage of the second class of tests is that they are based on actual symptoms of depression rather than on the different physicochemical properties of antidepressants. The anhedonia characteristic is also associated with human depression (Tsankova et al., 2006).

### 3.1.3. Obesity is linked with depression

Obesity has been linked with depression in different meta-analyses, clinical and epidemiological studies (Zhong et al., 2010; Wit et al., 2010; Boutelle et al., 2010; Stunkard et al., 2003; Simon et al., 2010; McElroy et al., 2004; Faith et al., 2002; Luppino et al., 2010). However a major question in the field is whether obesity or depression can play a causative role in the development of the other one. On one side, studies have shown that obesity, defined as a BMI, could predict the development of depression (Roberts et al., 2003; Roberts et al., 2000). In accordance with this observation, chronically obese males have significantly higher rates of depression compared to non-obese males (Mustillo et al., 2003), and obese versus non-obese adolescent females are at increased risk for depression as well (Anderson et al., 2007).

On the contrary, in other studies, initial high BMI was not related to the risk for future onset of depression (Stice and Bearman, 2001; Stice et al., 2000). Another study in elderly Koreans showed that obese elderly women were less likely to develop depression compare to their normal weight counterparts (Kim et al.,
Depression can promote the development of visceral obesity (Needham et al., 2010) and perturbed lipid metabolism (Rudisch and Nemeroff, 2003). In support of that, depression in youth positively correlates with the development of increased BMI during adulthood (Goodman and Huang, 2002; Pine et al., 1997; Pine et al., 2001; Richardson et al., 2003), suggesting a causative role of depression for obesity. Therefore, even though there is a link between obesity and depression, which of the two plays a causative role for the development of the other is still unknown, and understanding the molecular and cellular pathways that link obesity and depression is clearly of social and clinical importance and deserves further investigation.

### 3.1.4. Aims

Investigating diet as a risk factor for depression in humans is difficult because diet interacts with other environmental (stress) and biological (genetic) factors that can influence the incidence of depression in the population. The interaction of diet and depression is easier to study in mice, due to the more homogeneous environment in which they are raised/bred. Given the coprevalence of obesity and depression in different epidemiological studies, it was hypothesized that obesity (either dietary or genetic) has a mechanistic role in the development of a depression phenotype in mice.

The aims of this chapter are as follows:

1) To assess whether obesity in mice has a causative role in promoting the development of a depression phenotype

2) To investigate which molecular signalling pathways are altered after HFD induced obesity
3.2. Results

3.2.1. Dietary obesity induces a depression phenotype in mice

The diet-induced obesity (DIO) model was employed to study the role of dietary obesity in the development of depression. Male C57Bl/6J mice were used throughout the study as it has been shown that male mice are more vulnerable than females to high fat diet-induced obesity, metabolic alterations and deficits in learning and synaptic plasticity (Hwang et al., 2010). Moreover, male mice were used throughout the study as the menstrual cycle of females can affect performance during behavioural assays. Mice were fed either normal diet (ND) or a high fat diet (HFD), where 60% of calories are fat derived, to induce obesity. Consumption of a 60% fat diet was used as it has already been shown that consumption of 60% and not 41% fat impaired the cognitive function in mice and was linked with brain inflammation even though both diets lead to increase body weight (Pistell et al., 2010).

Exposure to HFD induces weight gain and metabolic syndrome, which develops after 6-8 weeks on HFD (Hwang et al., 2010). Table 3.1 shows the fatty acid composition of the HFD.
Table 3.1. Fatty acid composition of the HFD. Table shows the different fatty acid composition of the 60% diet that the mice consumed.

<table>
<thead>
<tr>
<th>HFD</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lard</td>
<td>245</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>25</td>
</tr>
<tr>
<td>C2, Acetic</td>
<td>0</td>
</tr>
<tr>
<td>C4, Butyric</td>
<td>0</td>
</tr>
<tr>
<td>C6, Caproic</td>
<td>0</td>
</tr>
<tr>
<td>C8, Caprylic</td>
<td>0</td>
</tr>
<tr>
<td>C10, Capric</td>
<td>0</td>
</tr>
<tr>
<td>C12, Lauric</td>
<td>0</td>
</tr>
<tr>
<td>C14, Myristic</td>
<td>2.2</td>
</tr>
<tr>
<td>C14:1, Myristoleic</td>
<td>1.2</td>
</tr>
<tr>
<td>C16, Palmitic</td>
<td>58.7</td>
</tr>
<tr>
<td>C16:1, Palmitoleic</td>
<td>9.3</td>
</tr>
<tr>
<td>C18, Stearic</td>
<td>33.5</td>
</tr>
<tr>
<td>C18:1, Oleic</td>
<td>106.8</td>
</tr>
<tr>
<td>C18:2, Linoleic</td>
<td>34.4</td>
</tr>
<tr>
<td>C18:3, Linolenic</td>
<td>4.4</td>
</tr>
<tr>
<td>C18:4</td>
<td>0</td>
</tr>
<tr>
<td>C20, Arachidic</td>
<td>4.4</td>
</tr>
<tr>
<td>C20:1</td>
<td>0</td>
</tr>
<tr>
<td>C20:4, Arachidonic</td>
<td>4.2</td>
</tr>
<tr>
<td>C20:5</td>
<td>0</td>
</tr>
<tr>
<td>C22, Behenic</td>
<td>0</td>
</tr>
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<td>C22:1, Erucic</td>
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</tr>
<tr>
<td>C22:4, Clupanodonic</td>
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</tr>
<tr>
<td>Total (g)</td>
<td>254.8</td>
</tr>
</tbody>
</table>
Depression related behavioural tests were conducted to assess the development of behavioural phenotype. Tail suspension test (TST), forced swim test (FST) and sucrose preference test (SPT) were used to assess the depression phenotype in mice. The elevated plus maze (EPM) was used in order to assess the anxiety phenotype in mice. Mice were tested at 2 different time points on the HFD; 3 weeks (a point prior to the development of the metabolic syndrome) and 8 weeks (after which mice have started to develop the metabolic syndrome). Fig. 3.1 is a schematic representing the experimental plan of the dietary induced obesity tests.

**Figure 3.1.** Schematic of the experimental plan for the dietary induced obesity and series of behavioral tests. Adult 10 weeks old male mice were fed HFD for either 3 or 8 weeks. At the end of each time point difference behavioural tests were performed in a specific order; first the anxiety related tests and then the depression related tests. (EPM: Elevated Plus Maze, OF: Open Field, TST: Tail Suspension Test, FST: Forced Swim Test, SPT: Sucrose Preference Test, d: day, wks: weeks).
Surprisingly, induction of depression, as assessed by increased immobilization time in the TST and FST, was observed as early as 3 weeks on HFD prior to the development of metabolic syndrome (Fig. 3.2). Similar effects of the HFD on TST and FST was observed between mice fed HFD for either 3 or 8 weeks suggesting that the depression phenotype happens early and remains the same over time (Fig. 3.2).

**Figure 3.2.** Dietary obesity induces a depression behaviour in mice. A) Tail suspension and B) forced swim test for WT mice that have been 3 and 8 weeks on ND, 3 and 8 weeks on HFD (n=10, *P < 0.05, **P < 0.01 by Linear mixed model fit by REML, Formula : immobilization ~ diet * time + (1 | ID). No interaction detected between diet and time and there was no effect of time on the immobilization time. Diet has an effect on the immobilization time). This work was carried out with Dr Jae Kyu Ryu.
HFD consumption was accompanied with less sucrose solution consumption compared to WT aged-matched controls on ND suggesting the occurrence of anhedonia and thus depression like behaviour (Fig. 3.3).

**Figure 3.3.** Dietary obesity induces a depression behaviour in mice. Sucrose preference test for WT mice on 3 and 8 weeks on HFD and their control aged matched mice on ND (n=5-8, **\( P < 0.01 \) by Linear mixed model fit by REML, Formula: sucrose ~ diet + (1 | time). Diet has an effect on the sucrose consumption).
As expected, WT mice on HFD gained significantly more weight compared to their aged matched control mice on ND as early as the first week on HFD (Fig. 3.4).

Figure 3.4. Body weight curves of WT mice on either ND or HFD. Comparison of the increase in the body weight of WT mice on HFD for 10 weeks and their litter mate mice on ND (n=10, **P < 0.01 by Linear mixed model fit by REML, Formula: weight ~ time + diet + time:diet + weight0 + (1 | id). An interaction was detected between diet and time (**P<0.001). Time (**P<0.001) and diet (**P<0.01) have an effect on the body weight. The body weight at time zero was used as a covariate and had no effect of the body weight gain).
Therefore, due to the body weight gain it is difficult to determine whether dietary obesity alone is a depression promoter. Interestingly, correlation graphs between immobilization time (TST and FST) and body weight showed that the performance of these mice was not affected by the increase in body weight either on ND or 3 weeks on HFD (Fig. 3.5). For that experimental design the mice were weighted just before they undergo the TST and FST, however a body weight change should also be plotted in relation to performance in the behavioural assays.

**Figure 3.5.** Correlation graphs between immobilization time and body weight. Correlation graphs between body weight and performance at the tail suspension and forced swim test for WT mice on either ND or 3 weeks on HFD (n=25-29). Analysis for carried out with Dr Jae Kyu Ryu.
Due to the observed depression effect of the HFD consumption, an earlier time point was tested in order to investigate the role of diet in depression after 1 week on the diet. Interestingly, a pilot study on mice just 1 week into a HFD regime showed similar depression phenotype compared to WT mice on ND (Fig. 3.6).

Figure 3.6. High fat diet induces a depression phenotype in mice. Tail suspension test for WT mice on ND and 1 week on HFD (n=9, *P < 0.05 by unpaired 2 tail unpaired student’s t-test).
3.2.2. Genetic obesity induces a depression phenotype in mice

To confirm these results in a genetic model of obesity, we examined whether genetic obesity also induces a depression phenotype. The leptin-deficiency mouse (Lep$^{ob}$ or ob/ob) was used as a model of genetically induced obesity (GIO). The leptin-deficiency mouse has been shown to spontaneously develop obesity even when kept on normal diet. Fig. 3.7 shows a schematic for the experimental plan for the GIO experiments.

Figure 3.7. Schematic of the experimental plan for the genetic obesity and series of behavioural tests. Adult 8 weeks old ob/ob male mice on ND were tested on different behavioural tests in a specific order; first the anxiety related tests and then the depression related tests. (EPM: Elevated Plus Maze, OF: Open Field, TST: Tail Suspension Test, FST: Forced Swim Test, SPT: Sucrose Preference Test, d:day, wks:weeks).
8 week old \textit{ob/ob} mice exhibited an increase in immobilization time in TST and FST compared to WT aged-matched mice on ND (Fig. 3.8).

![Tail Suspension Test and Forced Swim Test for WT and ob/ob mice on ND](image)

**Figure 3.8. Genetic obesity induces a depression behaviour in mice.** Tail suspension and forced swim test for WT and \textit{ob/ob} mice on ND (n=8-10, *\(P < 0.05\), ***\(P < 0.0001\) by unpaired 2 tail student’s t-test). This work was carried out with Dr Jae Kyu Ryu.

The immobilization values for the 8 week old \textit{ob/ob} mice on ND were very similar to the WT mice that were for 3 weeks on HFD (Fig. 3.2). As expected \textit{ob/ob} mice gained significant more weight even from the third week of life on ND compare to WT mice on ND (Fig. 3.9).

![Body weight curves of WT and ob/ob mice](image)

**Figure 3.9. Body weight curves of WT and ob/ob mice.** Comparison of the increase in the body weight of the \textit{ob/ob} mice compare to WT aged matched controls (n=6-11, ***\(P < 0.001\) by Linear mixed model fit by REML, Formula: weight ~ time + genotype + time:genotype + weight0 + (1 | id). An interaction was detected between genotype and time (***(P<0.001)). Genotype (***(P<0.001) and time (***(P<0.001) have an effect on the body weight. The body weight at 3 weeks of age was used as a covariate and had no effect on the body weight gain).
3.2.3. Dietary obesity does not interfere with the locomotor or rearing activity of mice

The TST and FST tests used in this study to assess depression are based on the motor performance of mice. However, consumption of the HFD and the subsequent increase in the body weight might affect the performance of mice to these tests and contribute to the depression phenotype. Therefore, the total locomotor activity of all mice, ND, HFD, ob/ob and WT, was tested at the open field test. Neither short nor longer HFD consumption affected locomotor or rearing activity of mice in the open field test (Fig.3.10). There was no significant difference in the total activity, time spent in the central area and rearing activity between ND and the two different time points (3 and 8 weeks) on the HFD. Rearing activity, which represents the exploratory activity of mice, was also not altered after HFD. This suggests that the development of the depression phenotype happened independently of any locomotor activity impairment.
3.2.4. Genetic obesity induces a reduction in the locomotor and rearing activity of mice

Genetically obese mice, *ob/ob* showed a reduction in the total locomotor and rearing activity in the open field test compared to WT aged matched control mice (Fig. 3.10). This mouse model of obesity has impaired total levels of activity as well as high anxiety levels indicated by the little time it spends in the central area. The exploratory activity of this mouse is also impaired as is shown by the total rearing activity measured by the open field test (Fig. 3.10).

![Graphs showing activity levels](image)

**Figure 3.10.** Genetic but not dietary obesity affects the locomotor activity. Open field test for WT mice on either ND or 3 and 8 weeks on HFD as well as *ob/ob* mice on ND. Measurements for total activity in the entire or central area as well as rearing (*n*=7-10 **P<0.01, ****P<0.0001, 1-WAY ANOVA with Tukey’s multiple comparison test).
3.2.5. Gene expression changes induced by HFD consumption

To investigate which specific molecular pathways in the brain are affected by a high-fat diet, comparative genome-wide microarray analysis was performed on samples from the hypothalamus of WT mice fed ND versus either 4 or 8 weeks on a HFD. The analysis was done on the hypothalamic region of the brain as it is known for its dual role in obesity and depression. Analysis of microarray data revealed that there were no significant gene expression differences between ND and 4 weeks on HFD. Interestingly, 8 weeks on high fat diet resulted in statistically highly significant changes in a number of genes. Table 3.2 shows all the significant gene expression changes between ND and 8 weeks on HFD as well as the values for these genes on 4 weeks on HFD. These are only the genes that have an average of log$_2$ intensity greater than 3.0 and an FDR (False Discovery Rate) value lower than 0.05. Even though many genes had a log$_2$ intensity greater than 3.0 the FDR value was not significant. The values for the WT mice on ND, 4 weeks and 8 weeks on HFD at the Table 3.2 are the average values for three samples per group. These values are the array values which are shown as log2 values after normalization.
Table 3.2. Gene expression changes in the hypothalamus of mice fed ND versus HFD. Table shows all the significant gene expression changes between ND and 8 weeks on HFD according to the FDR value as well as the values for these genes on 4 weeks on HFD. Each condition was done with 3 mice per group and average values are presented on the table.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Description</th>
<th>Entrez</th>
<th>WT ND</th>
<th>WT 4wks HFD</th>
<th>WT 8wks HFD</th>
<th>Week8 / CTRL</th>
<th>log2(Week8 / CTRL)</th>
<th>FDR Week8 vs. CTRL</th>
</tr>
</thead>
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<tr>
<td>Ptpn4</td>
<td>protein tyrosine phosphatase, non-receptor type 4</td>
<td>19258</td>
<td>8.6588</td>
<td>8.9779</td>
<td>7.5775</td>
<td>0.47</td>
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<tr>
<td>Serpina3n</td>
<td>serine (or cysteine) peptidase inhibitor, clade A, member 3N</td>
<td>20716</td>
<td>7.756</td>
<td>8.3121</td>
<td>9.0156</td>
<td>2.39</td>
<td>1.26</td>
<td>0.004</td>
</tr>
<tr>
<td>Nelf</td>
<td>nasal embryonic LHRH factor</td>
<td>56876</td>
<td>9.5734</td>
<td>9.8305</td>
<td>8.9099</td>
<td>0.63</td>
<td>-0.66</td>
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<td>0.24</td>
<td>-2.09</td>
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<td>adenylate cyclase 1</td>
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<td>q-Value</td>
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<td>regulator of G-protein signalling 7 binding protein</td>
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<td>Description</td>
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**Chapter 3** Obesity Induces Depression
A biocomputational approach was employed to aggregate genes which were up- or down-regulated in functional categories. The pathway analysis was performed using the QIAGEN’s Ingenuity® iReport (QIAGEN Redwood City, www.qiagen.com/ingenuity). Fig. 3.11 shows a heatmap of all the significant gene expression changes between ND and 8 weeks on HFD as well as the values for the 4 weeks on HFD and the top upregulated and downregulated genes after 8 weeks on HFD. Heatmap was constructed using the array values which are the log₂ values after normalization. Each condition was done with 3 samples.

**Figure 3.11.** HFD induces gene expression changes in the hypothalamus. Affymetrix global gene-expression analysis of hypothalamic samples on ND, 4 weeks and 8 weeks on HFD. Heat maps of the 68 genes with at least false DR less than 0.05 between ND and 8 weeks on HFD. The color range indicates the array values represented as log₂ after normalization. On the right are the top upregulated and downregulated genes that are affected by HFD.
Table 3.3 summarizes the 5 main canonical pathways and their p-values that were affected after exposure to HFD. Interestingly the most affected pathway from consumption of HFD in the hypothalamus was the protein kinase A (PKA) signalling pathway (Table 3.2).

Table 3.3. Top 5 main canonical pathways affected by HFD. The table summarizes the main canonical pathways and their p-values that were affected after exposure to HFD.

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<th>p-value</th>
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<td>Protein Kinase A signalling</td>
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<td>0,005070</td>
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Fig. 3.12 shows the main molecules at the PKA signalling pathway that were downregulated after the consumption of 8 weeks on HFD. The main molecules affected in the G protein coupled receptor and the GABA receptor signalling as also shown. Heatmap was constructing using the array values which are the log2 values after normalization.

HFD and regulate 3 main signaling pathways. The color range indicates the array values in the log2 scale after normalization.
Table 3.4. contains information about the main pathways that were affected by HFD with the relative genes and information about these genes. Fold change, p-value and false discovery rate analysis was done using the Ingenuity IPA software. In addition to PKA signalling, other pathways were also suppressed such as G-protein-coupled receptor or GABA receptor signalling involved in neuronal functions (Fig. 3.12 and Table 3.4).

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<th>Symbol</th>
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<th>p-value</th>
<th>False Discovery Rate (q-value)</th>
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<td>-1.637</td>
<td>2.49E-05</td>
<td>Cytoplasm</td>
<td>other</td>
<td></td>
</tr>
<tr>
<td>LEF1</td>
<td>Lymphoid enhancer-binding factor 1</td>
<td>-2.577</td>
<td>2.72E-06</td>
<td>Nucleus</td>
<td>transcription regulator</td>
<td></td>
</tr>
<tr>
<td>MYL2</td>
<td>Myosin, light chain 2, regulatory, cardiac, slow</td>
<td>-2.137</td>
<td>4.51E-05</td>
<td>Cytoplasm</td>
<td>other</td>
<td></td>
</tr>
<tr>
<td>PRKCD</td>
<td>Protein kinase C, delta</td>
<td>-5.952</td>
<td>1.30E-05</td>
<td>Cytoplasm</td>
<td>kinase</td>
<td></td>
</tr>
<tr>
<td>TCF7L2</td>
<td>Transcription factor 7-like 2 (T-cell specific, HMG-box)</td>
<td>-2.703</td>
<td>5.60E-05</td>
<td>Nucleus</td>
<td>transcription regulator</td>
<td></td>
</tr>
<tr>
<td>Epithelial adherens junction signaling</td>
<td>LEF1</td>
<td>lymphoid enhancer-binding factor 1</td>
<td>-2.577</td>
<td>2.72E-06</td>
<td>9.62E-03</td>
<td>Nucleus</td>
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<tr>
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</tr>
<tr>
<td>MYL2</td>
<td>myosin, light chain 2, regulatory, cardiac, slow</td>
<td>-2.137</td>
<td>4.51E-05</td>
<td>2.80E-02</td>
<td>Cytoplasm</td>
<td>other</td>
</tr>
<tr>
<td>TCF7L2</td>
<td>transcription factor 7-like 2 (T-cell specific, HMG-box)</td>
<td>-2.703</td>
<td>5.60E-05</td>
<td>3.07E-02</td>
<td>Nucleus</td>
<td>transcription regulator</td>
</tr>
<tr>
<td>GABA receptor signaling</td>
<td>GABRA4</td>
<td>gamma-aminobutyric acid (GABA) A receptor, alpha 4</td>
<td>-2.141</td>
<td>2.59E-05</td>
<td>2.27E-02</td>
<td>Plasma Membrane</td>
</tr>
<tr>
<td>GABRB2</td>
<td>gamma-aminobutyric acid (GABA) A receptor, beta 2</td>
<td>-1.541</td>
<td>9.95E-05</td>
<td>4.40E-02</td>
<td>Plasma Membrane</td>
<td>ion channel</td>
</tr>
</tbody>
</table>
Probing the hypothalamic protein samples of mice with a phospho-PKA substrate antibody that recognizes sites on proteins containing classical phosphorylated PKA sites (sequence RRXS, where S is phosphorylated) revealed a decrease of the total p-PKA immunoreactivity after HFD consumption compared to ND (Fig. 3.13).

![Western blot analysis using the phospho-PKA substrate that recognizes RRXS*/T* from mice fed ND versus 3 weeks on HFD. GAPDH was used as a loading control (n=4, ****P < 0.0001 by 2 tail unpaired student’s t-test).](image)

**Figure 3.13.** High fat diet downregulates the total p-PKA levels in the hypothalamus. Western blot analysis using the phospho-PKA substrate that recognizes RRXS*/T* from mice fed ND versus 3 weeks on HFD. GAPDH was used as a loading control (n=4, ****P < 0.0001 by 2 tail unpaired student’s t-test).

A major regulator of the PKA signalling is the ubiquitous second messenger cAMP. Because of the role of the cAMP signalling in depression (Nestler et al., 2002a) (Shelton, 2007) (Krishnan and Nestler, 2008) we assessed the effects of high fat diet on cAMP levels. cAMP measurements of hypothalamic samples derived from mice revealed a significant downregulation of cAMP levels after HFD consumption compared to ND (Fig. 3.14).
Chapter 3

Obesity Induces Depression

Figure 3.14. High fat diet downregulates the cAMP levels in the hypothalamus. cAMP measurements of hypothalamic samples of WT mice either on ND or 4 weeks on HFD (n=9-10, *P < 0.05 by 2 tail unpaired student’s t-test). One mouse from the ND group was excluded as it was a significant outlier according to the extreme studentized deviate or Grubb’s test.

3.3. Discussion

Despite the high comorbidity between obesity and depression shown in different epidemiological studies (Zhong et al., 2010; Leonore de Wit and Frans Zitman, 2010; Kerri N Boutelle, 2010; Boutelle et al., 2010), it has been difficult to determine which of the two plays a causative role for the development of the other due to the mixed results from the various studies. Moreover, little is known about the exact molecular pathways that link obesity with depression. Thus, with these gaps in our understanding we investigated whether obesity, either dietary or genetic, plays a causative role in the development of depression and which cellular signalling cascade underpins this phenomenon. Therefore, we specifically evaluated the role of diet in depression using male C57Bl/6J mice consuming HFD (diet-induced obesity) and the ob/ob mice (genetic obesity). Moreover, mRNA array analysis was performed to shed light on the molecular pathways that are influenced in the hypothalamus which is a major brain region involved in both obesity and depression after the consumption of a HFD.
3.3.1. Dietary or genetic obesity is sufficient to induce a depression phenotype in mice

The work in this study was done in vivo using WT mice that were fed either ND or HFD as well as ob/ob mice on ND. Using behavioural paradigms, we demonstrated that dietary or genetic obesity induces a depression phenotype in mice.

Therefore, the clinical data from the different patient studies (Zhong et al., 2010; Leonore de Wit and Frans Zitman, 2010; Boutelle et al., 2010; Stunkard et al., 2003; Simon et al., 2010; McElroy et al., 2004; Faith et al., 2002; Luppino et al., 2010) are in accordance with our current findings and strongly support a causative role of the western dietary habits and obesity with depression.

Interestingly, depression occurs independently of increases in body weight (Fig. 3.5). This observation is in agreement with epidemiological studies where normal weight people also develop depression (Kim et al., 2010). It has also been noticed that the relationship between immobility at the different behavioural tests and body weight in mice is not straightforward and cannot be taken as a priori fact (Collin et al., 2000). Moreover, preliminary experiments have shown no relationship between body weight and immobility at the depression related tests in mice (Collin et al., 2000). In agreement with that, high fat diet consumption was accompanied with a depression phenotype and altered serum lipids in cynomolgus monkeys with a surprisingly negative correlation between body weight gain, BMI and depression (Chilton et al., 2011). At the same study they also observed that only a subgroup of the monkeys develop a depression phenotype which means that western diet can increase depressive behaviour among a subgroup of susceptible individuals (Chilton et al., 2011). Moreover, several studies in humans found that BMI accompanied with impaired quality of life was positively associated with symptoms of depression (Dixon et al., 2003; Doll et al., 2000; Kolotkin et al., 2002). However when the impairments in health related quality of life were included in multivariate models there was no correlation between BMI and depression (Fabricatore et al.,
2005) further supporting the negative correlation between body weight and depression phenotype.

In the past, the role of HFD in mood disorders has been conflicting. In our study HFD has been shown to play a causative role in the development of a depression phenotype via signalling in the hypothalamus. However, HFD has been described to improve the depression behavioural profile and stress responses in male rats exposed to early life stress and this effect is mediated via the hippocampus (Maniam and Morris, 2010b; Prasad and Prasad, 1996). However Maniam et al used a diet comprising 32% originating from fat, as opposed to our study where 60% was deployed, which could also explain the opposing behavioural alterations. Therefore, different % of HFD might have a differential effect on different signalling pathways in different brain regions.

A caveat of this study is that the HFD used here consisted of 60% calories from fat content. This resulted in a 20% reduction of the carbohydrate content compared to the ND (Table 3.5). Interestingly, it has been shown that the effect of different fatty acids in the brain is dependent on the extracellular glucose levels in the hypothalamus suggesting an interaction between glucose and fatty acids (Wang et al., 2006). The apparent detrimental effect of HFD in depression phenotype in our study could therefore also be caused by the reduction of the carbohydrate in addition to the high fat content. Further studies are needed to investigate whether this could be the case.

Table 3.5. Percentage of main components of ND and HFD. Table summarizes the percentages of the fat, carbohydrates and protein that either the normal and high fat diet consists of.

<table>
<thead>
<tr>
<th></th>
<th>Normal Diet</th>
<th>High Fat Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>13.2%</td>
<td>60%</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>62.1%</td>
<td>20%</td>
</tr>
<tr>
<td>Protein</td>
<td>24.7%</td>
<td>20%</td>
</tr>
</tbody>
</table>
3.3.2. Dietary obesity induces a depression phenotype in mice

In this study mice were placed on a HFD for either chronic or short consumption and that was sufficient to induce a depression phenotype (Fig. 3.2). This observation is in agreement with a previous study shown that long time consumption of a HFD (16 weeks) induces a depression phenotype to mice compared to a normal diet (Yamada et al., 2011). However, here we show that just 3 weeks on a HFD was sufficient to induce a depression phenotype in vivo (Fig. 3.2). Moreover Yamada et al used mice between 12-14 weeks old but we used 8 weeks old mice. They further showed that substitution of the HFD with ND after the 16 week of HFD consumption ameliorated the depression phenotype in mice (Yamada et al., 2011). Whether the substitution of the HFD with ND in our study would reverse the depression phenotype remains to be determined.

Moreover, in a recent study diet-induced obesity promotes depressive phenotype in mice and it was suggested to be associated with neural adaptations in brain reward circuitry (Sharma and Fulton, 2013).

The FSL rat which is an inbred genetic model of depression has been shown to exhibit exacerbation of their depression phenotype after consumption of a HFD even though their counterparts were consuming higher caloric intake (Abildgaard et al., 2011). Since FSL is an inbred genetic model of depression, these findings strongly support an interaction between genetic and environmental factors, apparently rendering the depressive rat more easily influenced by dietary macronutrient composition. This is in agreement with epidemiological studies that there is genetic predisposition in the development of depression. However, what has not been shown before was whether a high fat diet can provoke depression-like behaviour. This study revealed for the first time that early consumption of the HFD can be a causative factor for the development of depression.
3.3.3. Genetic obesity induces a depression phenotype

The leptin deficiency ob/ob mouse in this study exhibit a depression phenotype (Fig. 3.8) which is in agreement with previous studies (Collin et al., 2000; Yamada et al., 2011). Leptin overexpression as well as leptin administration has been shown to have an antidepressant effect in mice (Yamada et al., 2011; Liu et al., 2010) suggesting that depression associated with obesity is partially due to impaired leptin action in the brain (Yamada et al., 2011). However, these studies have focused on the antidepressant role of leptin in the hippocampus (Yamada et al., 2011; Lu et al., 2006) as hippocampus is known for its role in depression (Sahay and Hen, 2007). Interestingly, selective deletion of the leptin receptor in the dopamine neurons did not induce any depression related behaviours in mice (Liu et al., 2011) suggesting that dopamine neurons are not implicated in the role of leptin in depression.

Leptin has been shown to affect cognition and mood in the cerebral cortex and hypothalamus (Harvey, 2007) however the neuronal populations as well as the neuronal circuits that mediate mood and are associated with the limbic functions of leptin remain to be clarified.

Another interesting point is that recent studies have demonstrated that consumption of dietary fats promotes hypothalamic resistance to the main anorexigenic hormones, leptin and insulin, leading to the progressive loss of balance between food intake and thermogenesis and therefore resulting in body mass gain (Milanski et al., 2009; De Souza et al., 2005; Munzberg et al., 2004). Moreover, leptin administration for the dietary induced obese mice did not induce any antidepressant effect (Yamada et al., 2011) suggesting the existence of another pathway different than leptin. Measurements of the anorexigenic and orexigenic hormones will be necessary to pinpoint their role in this obesity induced depression model.

Depression is characterized by an abnormality in the serotonergic system (Mann et al., 1996). Leptin deficient ob/ob mice have been shown to have decreased 5-HT transporter mRNA in the neurons of the dorsal raphe nucleus compare to WT
mice suggesting to play a role for the depression phenotype that they exhibit (Collin et al., 2000).

3.3.4. The development of the depression phenotype by HFD is independent of anxiety behaviour

The development of the depression phenotype due to the consumption of the HFD was not caused by the onset of anxiety as shown by the open field test data (Fig.3.10). However, in this study it was shown that the ob/ob mice exhibit an anxiety phenotype at the open field test compared to the WT mice (Fig.3.10). This is in agreement with previous literature as it has been shown that ob/ob mice exhibit increased anxiety (Finger et al., 2010) which was ameliorated when the ob/ob mice were treated with leptin (Asakawa et al., 2003). In accordance, it has been shown that acute administration of leptin leads to an anxiolytic effect (Liu et al., 2010).

3.3.5. PKA signalling in the hypothalamus was affected after dietary induced obesity

Protein phosphorylation by protein kinases and the dephosphorylation by protein phosphatases represent one of the major mechanisms of signal integration in eukaryotic cells. A kinase with a major role in cell signalling transduction is the protein kinase A (PKA). PKA is a tetrameric enzyme containing both regulatory and catalytic subunits (Gibson et al., 1997; Walsh et al., 1968). cAMP is bound by the regulatory subunits and this in turn, promotes dissociation of the active monomeric catalytic subunits, which are then free to phosphorylate other protein targets. PKA carries out its function by binding to its substrate and transferring one γ-phosphate group from ATP to its protein substrate. PKA activity is specific to the phosphorylation of serine/threonine residues located within an RxxS/T motif (Taylor et al., 2004; Daniel et al., 1998). PKA mediated phosphorylation of key signalling proteins represent a highly efficient kinetic amplification mechanism in neurons for the regulation of diverse pathways.
The mRNA array data in this study revealed the important role of the PKA signalling in the hypothalamus after the consumption of a HFD (Fig. 3.12). The finding that HFD downregulates the cAMP/PKA signalling cascade in the hypothalamus is of particular significance. Depression is in many ways a “pleiotropic” disorder that involves disturbances in mood, sleep, appetitive, immune and many other functions. PKA signalling exerts “pleiotropic” actions by means of phosphorylation of a variety of receptors, enzymes and transcription factors, proteins that are involved in neurotransmitter synthesis, regulation of G-protein coupled neurotransmitter receptors, and transcription factors such as cAMP responsive element binding protein (CREB) (Colbran et al., 1992). Many of these proteins that are regulated by PKA have known functions in depression.

The link between the PKA signalling cascade and depression has been documented (Perez et al., 2001). Chronic administration of antidepressant treatment or electroconvulsive seizures regulate PKA signalling in the brain (Menkes et al., 1983; Nestler et al., 1989b; Perez et al., 1989; Ozawa and Rasenick, 1991). These treatments have been shown to reduce cytosolic PKA while increasing PKA in the nucleus of rat frontal cortex samples (Nestler et al., 1989a). This suggests that antidepressant drugs or electroconvulsive seizures may stimulate the translocation of PKA from the cytosol to the nucleus. Antidepressant treatment and electroconvulsive seizures are believed to cause alterations in gene expression and as PKA regulates many transcription factors their action might be mediated via the PKA signalling. However, which genes of the PKA signalling cascade are involved has not been shown. In the same context, it has been shown that patients with major depression exhibit significantly less activity of β-adrenoceptor-linked protein kinase A than normal subjects (Shelton et al., 1996).

Here, we have identified genes in the PKA signalling cascade that are affected after administration of a HFD (Table 3.4) and these may play an important role in the development of depression. As protein phosphorylation by PKA has been proposed as a final pathway in the regulation of neuronal function, abnormalities in the PKA activity of depressed patients could have a wide range of cellular consequences (Shelton et al., 1996).
In addition, one of the genes that was downregulated after the consumption of the HFD is the protein kinase C delta (PRKCD) (Table 3.2). Using the ingenuity analysis software (www.qiagen.com/ingenuity), the ingenol 3-angelate (I3A) commonly named PEP005 is a potential drug that can be used in this study to reverse the depression phenotype. I3A is one of the active ingredients in *Euphorbia peplus*, which has been used in traditional medicine for its anticarcinogenic action. I3A has been shown to have a beneficial effect for the treatment of cancer (Ersvaer et al., 2010). A gel formulation of the drug has been approved by the U.S. Food and Drug Administration (FDA) and by the European Medicines Agency (EMA) for the topical treatment of actinic keratosis (Siller et al., 2009; Grue-Sorensen et al., 2014). I3A is a ligand and activator for the protein kinase C (PKC) (Kedei et al., 2004; Hampson et al., 2005).
This suggests that I3A might be able to reverse the depression phenotype observed after the consumption of HFD as HFD acts by reducing PKC signalling. Further investigation of the role of I3A in our model of depression is needed to investigate whether targeting specifically the PKC which is a downstream effector of PKA we can reverse the dietary induced depression phenotype.

**Figure 3.15** is a schematic of the main genes affected at the PKA signalling cascade after the consumption of the HFD and how they connect between each other.

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**Figure 3.15.** The main genes at the PKA signalling pathway affected by HFD. Schematic represents the main genes affected by HFD and how they connect between each other. Schematic was designed using the Ingenuity analysis software. With green color are the genes that were downregulated after HFD consumption.
3.3.6. The role of hypothalamus in the obesity induced depression phenotype

A 50% reduction of the total neurons in the paraventricular nucleus (PVN) in the hypothalamus has been observed in patients with major depression (Manaye et al., 2005). This study focused on the investigation of the signalling pathways affected after HFD in the hypothalamus (Fig. 3.11). However, other brain regions such as the frontal cortex, amygdala and hippocampus that have been studied extensively with regard to psychiatric disorders as they have been shown to be related to emotions, memory and executive function might play an important role as well. The role of the hypothalamus as well as cerebellum has been underemphasized and has been thought to play a minor role at best. It is, however, now becoming clear that the cerebellum plays a more important role than previously thought in major depression (Beauregard et al., 1998; Liotti et al., 2002; Smith et al., 2002). Data coming from this study also put emphasis on the role of hypothalamus in the development of obesity induced depression phenotype. Further studies investigating the other brain regions and their involvement in this model are necessary. However, even though in this study we were able to determine a role of hypothalamus in depression, the exact hypothalamic nuclei that are critical for the depression phenotype in response to diet remain to be determined.

3.3.7. The role of the HPA axis in the obesity induced depression phenotype

Dysregulation in many different pathways have linked obesity with depression. Impaired function of glucocorticoid receptors (Holsboer, 2000; Ljung et al., 2002) as well as disturbances in central serotonin, norepinephrine and dopamine neurotransmitter systems (Bjorntorp and Rosmond, 2000) have been identified to play a pivotal role. Moreover, the dysregulation of the hypothalamus-pituitary-adrenocortical (HPA) axis have also been involved in depression. Hypothalamus plays a pivotal role and regulates main functions of the HPA axis. Cytokines and adipokines (Elenkov et al., 2005; Miller et al., 2003) play an important role in pathophysiology of obesity and depression (Rajala and Scherer, 2003; Wisse, 2004) and interact with the HPA axis and the neurotransmitter systems.
(Besedovsky et al., 1986; Schiepers et al., 2005). Moreover overweight and obesity has been shown to impair the HPA-axis in depressed and nondepressed individuals (Vicennati and Pasquali, 2000), suggesting that increase in body weight can play a role. Moreover, leptin has been shown to suppress the HPA axis (Heiman et al., 1997). Therefore, further analysis of the HPA pathway in our dietary and genetically obesity induced depression phenotype should be investigated in order to shed light on the role of this pathway in depression caused by diet.

3.3.8. The role of neurogenesis in depression

HFD consumption has a major impact on the cellular architecture of the arcuate nucleus in the hypothalamus; it affects the structure of the BBB so that the POMC and NPY cell bodies and dendrites become less accessible to blood vessels (Horvath et al., 2010) and this effect might be irreversible due to reactive gliosis (Horvath et al., 2010). Moreover, HFD triggers a loss of synapses on POMC neurons in the hypothalamus (Horvath et al., 2010). Therefore, one possible route for the induction of depression after HFD is aberrant neuronal death. Hippocampal neurogenesis has been shown to have an antidepressant effect in mice and humans (Sahay and Hen, 2007). Moreover, various antidepressant treatments have been shown to increase hippocampal neurogenesis. Cell proliferation and de novo neurogenesis has been described in the hypothalamus to act as a compensatory mechanism to regulate energy balance during progressive degeneration of hypothalamic neurons (Pierce and Xu, 2010). It has already been described that consumption of a HFD for 8 weeks induces apoptosis of hypothalamic neurons and this effect is dependent upon diet composition and not caloric intake (Moraes et al., 2009). Therefore, further investigation whether hypothalamic neurogenesis is affected in this model of depression and determination of whether neurogenesis in specific hypothalamic nuclei is responsible for the dietary or genetic induced depression behavior is required. Nissl staining would reveal any cell loss or abnormalities in cell density and cytoarchitecture in the hypothalamus that might suggest the cellular toxicity. BrdU positive cells represent the number of survived cells (Schmidt and Duman, 2007).
3.4. Conclusions

In conclusion, dietary or genetic obesity plays a causative role in the development of depression in mice. Importantly, this happens independently of the increase in the body weight and at a very early stage of obesity. The downregulation of the cAMP/PKA signaling pathway in the hypothalamic area after the consumption of the HFD is of significant importance. Moreover, this study presents for the first time the identification of specific genes that were downregulated in the PKA signaling cascade in the hypothalamus after HFD. To our knowledge this is the first time that PKA signaling pathway is the linker molecular pathway in the hypothalamus that connects obesity and depression.
Chapter 4

The loss of PDE4A rescues the depression phenotype observed after dietary or genetic obesity
4. Introduction

4.1. Introduction

Antidepressant drugs are broadly prescribed for a variety of affective disorders; however, in many cases the molecular and cellular adaptations that underlie their therapeutic action remain unknown. It is also clear that the molecular targets for the development of therapeutic agents have remained the same the last 40 years (mainly the inhibition of monoamine metabolism and reuptake). Resistance to antidepressant treatments observed for overweight and obese people suggests that unique depression signalling pathways exist for this subpopulation (Papakostas et al., 2005). Identifying these pathways might shed light on the precise mechanism that links obesity with depression. This knowledge will help developing selective strategies for therapeutic intervention to treat depression that target molecular pathways specific for the overweight and obese subpopulation.

4.1.1. 3’, 5’- cyclic adenosine monophosphate signalling pathway in depression

3’, 5’-cyclic adenosine monophosphate (cAMP) is a ubiquitous second messenger that mediates signal transduction of several neurotransmitters including dopamine, norepinephrine, serotonin and adenosine, all of them targets of antidepressant treatment (Shelton, 2007). It has been proposed that the mechanism of action for most of the antidepressant treatments involve the upregulation of the 3’-5’-cyclic adenosine monophosphate (cAMP) cascade (Duman et al., 1997). cAMP is synthesized from adenosine 5’-triphosphate (ATP) by adenylyl cyclase (AC), and hydrolysed by anchored cAMP degrading cyclic nucleotide phosphodiesterases (PDEs). The increase in intracellular levels of cAMP at the inner surface of the plasma membrane can then activate downstream effectors such as protein kinase A (PKA), which in turn trigger downstream signals that lead to the regulation of various cellular processes including proliferation, differentiation and apoptosis (Tasken and Aandahl, 2004). Forskolin, a plant diterpene that directly activates adenylyl cyclase, is
reported to have antidepressant actions in depressed patients (Bersudsky et al., 1996).

Tight control of cAMP signalling is orchestrated by strict temporal regulation of the generation and degradation of this second messenger. This spatiotemporal control of the cAMP signalling dynamics is achieved by numerous feedback regulations that are activated to finely tune the cAMP signal in intensity, time and its propagation through the intracellular space. This process is centrally achieved by various proteins, including PKA, PDEs and is facilitated by scaffolding proteins such as A-kinase-anchoring proteins (AKAPs) (Bauman et al., 2007; Tasken and Aandahl, 2004; Wong and Scott, 2004). Of all the different PDEs, PDE4 has been shown to play a critical role for the cAMP signalling in the brain (Conti et al., 2003).

4.1.2. The role of phosphodiesterase inhibitors in depression

The sole route for cAMP degradation occurs via the PDEs. Preclinical studies have reported that PDE inhibitors have antidepressant like effects in animal models (Wachtel and Schneider, 1986; Griebel et al., 1991). Papaverine, a non-specific PDE inhibitor, was also found to have antidepressant action in a case report (Malison et al., 1997). Tadalafil, a PDE5 inhibitor has been shown to improve depressive symptoms, ameliorate memory impairments, suppress apoptosis and enhance cell proliferation of hippocampal cells in a model of depression, all of which are characteristics of an antidepressant effect (Baek et al., 2011). Resveratrol, a phosphodiesterase inhibitor which occurs naturally in the skin of red grapes, has been shown to improve memory performance and hippocampal neuronal functional connectivity (Witte et al., 2014), both of which are affected in people with depression.

Rolipram, a small-molecule and a generic PDE4 inhibitor, is known for its antidepressant action in rodents (Zhang, 2009; Zhang et al., 2006; Przegalinski and Bigajska, 1983; Overstreet et al., 1989; Schwabe et al., 1976; Zeller et al., 1984; Kehr W, 1985; Wachtel, 1982; Wachtel, 1983b) and in clinical trials (Griebe et al., 1991; Bobon et al., 1988; Wachtel and Schneider, 1986; Wachtel,
1983a; Fleischhacker et al., 1992; O'Donnell, 1993; Bertolino et al., 1988; Hebenstreit et al., 1989). However, despite initial promise, the therapeutic potential of rolipram as an antidepressant is limited due to the side effects of nausea, emesis (Scott et al., 1991; Zeller et al., 1984; Rock et al., 2009; Robichaud et al., 2001), owing to its mechanism of action as a non-isoform-specific PDE4 inhibitor (Dyke and Montana, 2002; Robichaud et al., 2002; Hirose et al., 2007; Bertolino et al., 1988; Hebenstreit et al., 1989). The molecular identification of the isoform that mediates the antidepressant action of rolipram and the creation of selective inhibitors of PDE4 isoforms may have therapeutic effects with minimal adverse reactions (O'Donnell and Zhang, 2004). Structural analysis of the catalytic domains of PDE4A, PDE4B, PPDE4C and PDE4D bound to inhibitors revealed significant conformational differences between the different enzymes that can shed light for the development of selective inhibitors for the different members of the PDE4 family (Wang et al., 2007). Despite the fact that the design of specific PDE4 inhibitors has been very slow, it has already been achieved for the sub-family of PDE4Ds in order to enhance cognition (Burgin et al., 2010; Houslay and Adams, 2010). However, no PDE4 inhibitor have yet been brought to market as an antidepressant because of issues related to tolerability and side effects (Giembycz, 2005).

4.1.3. The role of phosphodiesterase in depression

PDE4 gene family plays a major role in regulating the cAMP signalling in the brain (Houslay, 2001; Menniti et al., 2006) and is the primary family of phosphodiesterase hydrolysing cAMP associated with the central noradrenergic system that is involved in depression (Whalin et al., 1988; Challiss and Nicholson, 1990; Ye and O'Donnell, 1996). PDE4 isoforms show specific expression patterns (Houslay, 2001) suggesting their unique role in signalling. PDE4s have also been linked with many psychiatric disorders as these enzymes are localized in certain areas of the brain (Iona et al., 1998) that are associated with reinforcement, movement and affect (Cherry and Davis, 1999) all of which are affected in people with depression. In light of this data, PDE4 has been regarded as a potential therapeutic target for the treatment of various mental diseases including depression (Houslay et al., 2005; Millar et al., 2005).
Much of the published work has been done using the \textit{PDE4D}\textsuperscript{-/-} mice investigating the role of \textit{PDE4D} on memory in the hippocampus. \textit{PDE4D}\textsuperscript{-/-} mice display memory enhancement that was mimicked by repeated rolipram treatment in WT mice (Li et al., 2011). Memory enhancement was not affected for the \textit{PDE4D}\textsuperscript{-/-} mice by chronic antidepressant treatment, indicating the predominant role of \textit{PDE4D} in the mediation of memory (Li et al., 2011). Using miRNA infusion in the hippocampus the long isoforms of the \textit{PDE4D} were identified as responsible for memory enhancement, hippocampal neurogenesis and CREB phosphorylation, and appeared not to cause emesis (Li et al., 2011) suggesting their potential as memory enhancers without side effects. The \textit{PDE4D}\textsuperscript{-/-} mice exhibit antidepressant behaviour as assessed by TST and FST (Zhang et al., 2002). \textit{PDE4D}\textsuperscript{-/-} mice showed a reduced sensitivity to rolipram but not for other antidepressants such as desipramine or fluoxetine, suggesting \textit{PDE4D} is an essential mediator of the antidepressant effect of rolipram (Zhang et al., 2002).

From the different members of the PDE4 family, the expression of \textit{PDE4A} and \textit{PDE4B} subtypes, but not \textit{PDE4D}, specifically increases after repeated treatment with antidepressant drugs such as desipramine and fluoxetine (Takahashi et al., 1999; Ye et al., 1997; Ye et al., 2000). Upregulation of the \textit{PDE4} gene expression in response to sustained activation of the cAMP pathway (Conti et al., 1995; Conti and Jin, 1999) is thought to represent a compensatory adaptation in response to the antidepressant treatment that would reduce cAMP levels back to baseline (Conti et al., 1995; Houssay and Milligan, 1997). This possibility is supported by \textit{in vitro} studies where cAMP stimulation of \(\beta\)-adrenergic receptors or direct activation of adenylyl cyclase or cAMP dependent protein kinase increases the RNA that leads to PDE4A protein expression (Torphy et al., 1995; Manning et al., 1996; Verghese et al., 1995). Moreover, \textit{in vivo} studies reveal that \textit{PDE4A} mRNA and protein activity in the brain appear to be influenced by \(\beta\)-adrenergic receptor activation, as treatment with the \(\beta\)-adrenergic receptor antagonist decreases expression of this isoform (Ye and O'Donnell, 1996; Ye et al., 1997). However, the exact underlying mechanism for the upregulation of \textit{PDE4A} and \textit{PDE4B} expression in brain is unknown, but it is proposed to involve activation of gene expression in response to stimulation of the cAMP pathway (Conti et al., 1995; Conti and Jin, 1999).
From the different PDE4As, the long isoform of this gene family (PDE4A5) has been implicated in the regulation of different pathways. The N-terminal of PDE4A5 isoform confers an ability to interact with the SH3 domains of specific proteins, such as that of the SRC family tyrosyl kinase LYN (O’Connell et al., 1996) and this interaction affects its catalytic unit (McPhee et al., 1999). PDE4A5 has been shown to have a role in the survival of at least certain cells as it is the sole PDE4 isoform that is cleaved by caspase-3 during apoptosis causing it to be redistributed within the cell (Huston et al., 2000). PKA phosphorylation of PDE4A4 in humans, which is the equivalent of PDE4A5 in mice, increases its activity as well as the sensitivity to rolipram (Laliberte et al., 2002). PDE4A5 interacts with immunophilin XAP2 and this interaction reversibly inhibits the enzymatic activity, attenuates the ability of PKA to phosphorylate PDE4A5 and increases the sensitivity to rolipram (Bolger et al., 2003b). PDE4A5 has also been shown to be activated by stimulation of the PI-3 kinase cascade (MacKenzie et al., 1997). Very recently, PDE4A5 was shown to be the major isoform in the hippocampus responsible for the memory impairment due to sleep deprivation (Vecsey et al., 2009). Activation of PDE4A5 inhibits the differentiation of 3T3 cells to adipocytes (MacKenzie et al., 1998). All these previous studies clearly underlined the important role of PDE4A5 in the regulation of different molecular pathways.

In summary, a variety of studies have identified the cAMP signalling pathway to be involved in the depression phenotype, and have highlighted the importance of the different PDE4s as well as specific roles for individual PDE isoforms in cAMP/PKA signalling in depression.
4.1.4. Aims

Although there is a well-established link between obesity and depression, critical mechanistic insights that underpin the links between obesity and depression are still unknown. Given the fact that high fat diet downregulates PKA signalling in the hypothalamus it was hypothesized that PDE4, the major regulators of the cAMP signalling in the brain, and therefore, PKA modulators, may be involved in the regulation of PKA signalling to attenuate the depression phenotype. Hence, this chapter will focus on the role of PDE4s in dietary or genetic obesity induced depression.

The aims of this chapter are as follows:
1) to define whether phosphodiesterases play a role in the depression phenotype induced by dietary or genetic obesity
2) to identify which specific isoform(s) of the PDE4 family play a functional role in maintaining the link between obesity and depression

4.2. Results

4.2.1. Chronic but not acute administration of rolipram tend to reduce the dietary-induced depression phenotype in mice

As mentioned previously, rolipram is a generic PDE4 inhibitor with reported antidepressant action in mice. However, whether rolipram can rescue the dietary obesity-induced depression phenotype observed in this model is unknown. In mice on 3 weeks of HFD, acute intraperitoneal injection (i.p.) of rolipram did not affect the immobilization time of mice compared to saline injected mice (Fig. 4.1), suggesting that acute rolipram administration did not affect the dietary obesity-induced depression phenotype.
Figure 4.1. Acute rolipram injection has no effect on the dietary obesity induced depression phenotype. Tail suspension test for WT mice on 3 weeks on HFD i.p. injected with either saline or rolipram (n=4-5).

Next, chronic administration of rolipram was assessed for its effect on the dietary obesity-induced depression phenotype. Fluoxetine (Prozac) was used as a positive control due to its popularity as a widely prescribed antidepressant drug with known antidepressant action in mice, though as with rolipram, its ability to rescue the depression phenotype observed after HFD consumption had been previously untested. Immobilization time in the TST tended to be reduced in mice injected with rolipram or Prozac compared to control mice, injected with saline (Fig. 4.2). Even though the reduction of the immobilization time was not significant, it revealed a potentially effect that should be further investigated with bigger cohorts of mice as there were only 5 mice per group in that study.
Figure 4.2. Chronic administration of either prozac or rolipram reduces the immobilization time at the tail suspension test. Tail suspension test for WT mice on 3 weeks on HFD daily i.p. injected with either saline, rolipram or Prozac (n=5).
Prozac or rolipram administration increased sucrose consumption over water indicating the reversal of the anhedonia phenotype (Fig. 4.3), though again, this was not a statistically significant effect.

![Sucrose Preference Test](image)

Figure 4.3. Chronic administration of either prozac or rolipram increases the sucrose consumption. Sucrose preference test for WT mice on 3 weeks on HFD daily i.p. injected with saline, rolipram or Prozac (n=5).

The mice that received daily Prozac injections gained significantly more weight compared to the saline-injected mice, and this was observed as early as after the first injection (Fig. 4.4). Surprisingly, the opposite effect was observed with the rolipram-injected mice, as they were resistant to the dietary induced obesity phenotype observed for the saline injected mice (Fig. 4.5).
Figure 4.4. Daily Prozac injections increase body weight compare to saline injected mice. Body weight of WT mice injected daily i.p. with Prozac or normal saline for 4 weeks while on HFD (n=5, **P < 0.01 by Linear mixed model fit by REML, Formula: weight ~ time + drug + time:drug + weight0 + (1 | id). Time (**P<0.001) and drug (**P<0.01) have an effect on the body weight. The body weight at day 1 was used as a covariate and had no effect of the body weight gain).

Figure 4.5. Daily rolipram injections tend to decrease body weight compare to saline injected mice. Body weight of WT mice injected daily i.p. with either normal saline or rolipram for 4 weeks while on HFD (n=5, by Linear mixed model fit by REML, Formula: weight ~ time + drug + time:drug + weight0 + (1 | id). Time (**P<0.001) and the interaction between time and drug (**P<0.01) have an effect on the body weight. The body weight at day 1 was used as a covariate and had no effect of the body weight gain).
In addition, liver measurements revealed an increase in liver weight of the mice daily injected with Prozac compare to either saline or rolipram (Fig. 4.6).

![Liver weight graph](image)

**Figure 4.6.** Chronic administration of Prozac increases liver size compare to either saline or rolipram. Liver weight for WT mice on 3 weeks on HFD daily i.p. injected with either saline, rolipram or Prozac (n=5, **P<0.01 by 1-way ANOVA following by Dunnett’s multiple comparison test).**
4.2.2. High fat diet specifically increases phosphodiesterase 4 (PDE4) activity in the hypothalamus of WT mice

Since the PKA signalling pathway is down regulated after the consumption of HFD (Chapter 1, Fig. 3.12 & 3.13), it was hypothesized that this effect might happen via the regulation of phosphodiesterase activity. That was further supported by the effect of daily rolipram injections, though again, this was not a statistically significant effect (Fig. 4.2 & 4.3). Phosphodiesterases are major regulators of PKA signalling pathways as they play a pivotal role in the temporal and spatial regulation of cAMP. In light of this, I tested whether high fat diet alters the activity of PDE4 in the brain. Four brain regions have been shown to be involved in depression: hypothalamus, amygdala, cortex and hippocampus (Price and Drevets, 2010). Interestingly, using biochemical assays, a significant increase of PDE4 activity of hypothalamic samples of WT mice fed HFD for 3 weeks compared to mice fed ND was found (Fig. 4.7).

![Figure 4.7. HFD increases the levels of phosphodiesterase4 activity in the hypothalamus. PDE4 activity for whole lysates of hypothalamic samples of WT mice fed either normal diet or 3 weeks on HFD (n=7-10 unpaired two tail student’s t-test *P<0.05). This work was carried out by Dr Jonathan Day.](image-url)
This effect was specific for the hypothalamus as HFD did not alter the total PDE4 activity levels in cortex (Fig. 4.8), tended to decrease but not significantly in the hippocampus (Fig. 4.9), did not affect the cerebellum (Fig. 4.10) and tended to increase but not significantly in amygdala (Fig. 4.11) further proving that the hypothalamic area is the only area where the PDE4 activity is affected by HFD.

Figure 4.8. HFD does not alter the levels of phosphodiesterase activity in the cortex. PDE4 activity for whole lysates of cortical samples of WT mice fed either normal diet or 3 weeks on HFD (n=11) This work was carried out by Dr Apostolos Zarros.

Figure 4.9. HFD does not alter the levels of phosphodiesterase activity in the hippocampus. PDE4 activity for whole lysates of hippocampal samples of WT mice fed either normal diet or 3 weeks on HFD (n=9-10). This work was carried out by Dr Apostolos Zarros.
Figure 4.10. HFD does not alter the levels of phosphodiesterase activity in the cerebellum. PDE4 activity for whole lysates of cerebellum samples of WT mice fed either normal diet or 3 weeks on HFD (n=9-11) This work was carried out by Dr Apostolos Zarros.

Figure 4.11. HFD does not alter the levels of phosphodiesterase activity in the amygdala. PDE4 activity for whole lysates of amygdala samples of WT mice fed either normal diet or 3 weeks on HFD (n=7-11). This work was carried out by Dr Apostolos Zarros.
4.2.3. High fat diet alters the RNA and protein levels of PDE4A5 in the hypothalamus of WT mice

In mice and humans, the PDE4 gene family is comprised of the PDE4-A, -B, -C and -D genes, each of which gives rise to more than four different protein isoforms (Perez-Torres et al., 2000). The further study of the PDE4C sub-family was excluded as it has been shown that it is not expressed in the brain (Perez-Torres et al., 2000; Swinnen et al., 1989; Iona et al., 1998). Real-time PCR analysis for total RNA levels of PDE4B in the hypothalamus of mice showed undetectable RNA levels (data not shown). There was no significant difference in the total RNA levels of PDE4D in mice fed normal versus HFD or the genetically obese mice with a trend to be downregulated after the consumption of HFD for one week (Fig.4.12).

Figure 4.12. Dietary or genetic obesity does not alter the RNA expression levels of PDE4D. Real-time PCR analysis of PDE4D in the hypothalamus of WT mice on ND, 1 week and 3 weeks on HFD and ob/ob mice (n=4)
Interestingly, there was an upregulation of the RNA levels for the PDE4A (Fig. 4.13). PDE4A gene produces different transcripts due to alternative splicing; one of them is the PDE4A5 that has been shown to be regulated by sleep deprivation (Vecsey et al., 2009). Surprisingly, there was a specific RNA increase for PDE4A5 in the hypothalamus of mice fed HFD compared to ND (Fig. 4.13).

Figure 4.13. Dietary or genetic obesity increases the RNA expression levels of PDE4A and PDE4A5. Real-time PCR analysis for PDE4A and PDE4A5 in the hypothalamus of WT mice on ND, 1 week and 3 weeks on HFD and ob/ob mice (n=4, *P < 0.05 , **P<0.01, by one-way ANOVA with Dunnett’s multiple comparison test).
Western blot analysis of tissue from the hypothalamus region of WT mice fed either ND or 3 weeks HFD revealed a significant upregulation of the protein levels of PDE4A5 in the hypothalamic area (Fig. 4.14) that was in agreement with the induction of RNA (Fig. 4.13).

![Western blot analysis for PDE4A5 from mice fed ND versus 3 weeks on HFD. GAPDH was used as a loading control (n=4, *P < 0.05 by 2 tail unpaired Student’s t test). Experiment was performed twice and representative blot with its quantification is shown.](image)

**Figure 4.14.** High fat diet increases specifically the PDE4A5 protein levels in the hypothalamus. Western blot analysis for PDE4A5 from mice fed ND versus 3 weeks on HFD. GAPDH was used as a loading control (n=4, *P < 0.05 by 2 tail unpaired Student’s t test). Experiment was performed twice and representative blot with its quantification is shown.
4.2.4. High fat diet leads to attenuation of CREB phosphorylation in the hypothalamus

The transcription factor CREB is a well characterized substrate for PKA that has been implicated in the mechanism of action of many antidepressant drugs. Probing hypothalamic samples of mice fed with HFD for 3 weeks, a decrease phosphorylation at Ser 133 of CREB was observed (Fig. 4.15) suggesting that exposure to a HFD results in an attenuation of PKA-mediated CREB phosphorylation.

Figure 4.15. High fat diet decreases the levels of CREB phosphorylation in the hypothalamus. Western blot analysis for total p-CREB and CREB protein levels from hypothalamic samples of mice fed ND versus 3 weeks on HFD. (n=3, **P < 0.01 by 2 tail unpaired Student’s t test).
4.2.5. *In vivo* loss of PDE4A gene rescues the dietary or genetically obesity-induced depression phenotype

If an upregulation of PDE4A5 activity was orchestrating cAMP dynamics that resulted in a decrease in phospho-CREB levels, it was hypothesized that the *in vivo* loss of PDE4A gene might rescue the dietary or genetically obesity-induced depression phenotype observed in this model. To test this hypothesis, the PDE4A\(^{-/-}\) mouse was utilised, (a kind gift from Professor Marco Conti, UCSF). The PDE4A\(^{-/-}\) and their littermates PDE4A\(^{+/+}\) were placed for 3 weeks on HFD. The double knock out *ob/ob*:PDE4A\(^{-/-}\) was also generated by crossing these two mouse lines together. This strain was used to test whether the loss of PDE4A can rescue the genetic obesity-induced depression phenotype. Loss of PDE4A rescues the dietary and genetically obesity induced depression phenotype as shown by the tail suspension and forced swim tests (Fig4.16).

![Tail Suspension Test](image1)

**Figure 4.16. Loss of PDE4A in vivo rescues the dietary or genetic induced depression phenotype.** Tail suspension and forced swim test for PDE4A\(^{+/+}\) and PDE4A\(^{-/-}\) mice on either ND and 3 weeks on HFD, *ob/ob* and *ob/ob*:PDE4A\(^{-/-}\) mice on ND (n=7-10, *P < 0.05, **P<0.01, ***P<0.001, ****P<0.0001 by one way ANOVA with Tukey’s multiple comparison test).
The $PDE4A^{-/-}$ and their $PDE4A^{+/+}$ litter mate controls showed the same increase in body weight on ND and HFD further proving that the depression phenotype does not correlate with the increase in body weight (Fig. 4.17 and Fig. 4.18). Similarly, the genetically obese ($ob/ob$) mice and the double knock-out $ob/ob:PDE4A^{-/-}$, exhibited comparable body weights on ND (Fig. 4.17).

![Figure 4.17](image1)

**Figure 4.17.** The loss of PDE4A does not induce any body weight changes on ND. Body weight curves of $PDE4A^{+/+}$, $PDE4A^{-/-}$, $ob/ob$ and $ob/ob:PDE4A^{-/-}$ on ND ($n=7-10$).

![Figure 4.18](image2)

**Figure 4.18.** Loss of PDE4A does not alter the increase in body weight after HFD consumption. Body weight curves of $PDE4A^{+/+}$, $PDE4A^{-/-}$ on HFD ($n=9-10$).
4.2.6. Genetic ablation of PDE4A is sufficient to abolish the increased PDE4 activity observed in hypothalamus

Previously, it was shown that HFD upregulates the total PDE4 activity levels in the hypothalamus compared to ND (Fig. 4.7). Phosphodiesterases regulate the spatiotemporal concentration of cAMP (Fig. 1.3). Therefore, further investigation was performed in order to elucidate in which subcellular compartment the increased PDE4 activity occurs. Using biochemical assays, the PDE4 activity was measured at the membrane and cytosol fraction of hypothalamic samples from mice fed ND and HFD. A specific increase in PDE4 activity in the membrane fraction (but not the cytosolic fraction) of hypothalamic tissue from mice fed high-fat diet for 3 weeks compared to ND was observed (Fig.4.19). That increase was not observed for the PDE4A<sup>−/−</sup> mice (Fig.4.19). As no differences were detected in the PDE4 activity of the cytosol fraction from either WT or PDE4A<sup>−/−</sup> on ND or HFD, I hypothesized that compartmentalization of the phosphodiesterase activity within the membrane fraction is critical for the fidelity of the cAMP/PKA signals, which underpin onset of the depression phenotype.

![Figure 4.19. High fat diet increases PDE4 activity at the membrane fraction of the hypothalamus that is rescued at the PDE4A<sup>−/−</sup> mice. PDE4 activity assay measurements at the membrane and cytosol fraction of hypothalamus of PDE4A<sup>+/+</sup> and PDE4A<sup>−/−</sup> on either ND versus 3 weeks on HFD (n=4-6, ***P < 0.001 by one-way ANOVA with Dunnett’s multiple post hoc test). This experiment was performed with Dr Apostolos Zarros.](image-url)
No differences were detected in the cytosol or membrane fractions of amygdala for the WT or $PDE4A^{-/-}$ on either ND or HFD, further proving the specificity of the effect of HFD on the hypothalamic area (Fig. 4.20).

Figure 4.20. High fat diet does not alter the PDE4 activity in amygdala either at the cytosol or at the membrane fraction. PDE4 activity assay measurements at the membrane and cytosol fraction of amygdala of PDE4A$^{+/+}$ and PDE4A$^{-/-}$ mice on either ND or 3 weeks on HFD (n=4-6).
Using a specific antibody that recognizes the PKA phosphorylation within the UCR1 of PDE4A5, an increased phosphorylation was observed for the hypothalamic samples that were fed HFD for 3 weeks compared to ND (Fig. 4.21). This phosphorylation is known to activate PDE4A5, so it is possible that the increase in PDE4 activity seen in the hypothalamic membrane fractions is the result of a combination between increased PDE4A5 protein (Fig. 4.14) and increased PDE4A5 activation (Fig. 4.21).

![Western blot analysis](image)

**Figure 4.21.** High fat diet increases the levels of phosphorylation of PDE4A5 in the hypothalamus. Western blot analysis for membrane hypothalamic samples of p-PDE4A5 from mice fed ND versus 3 weeks on HFD. GAPDH was used as a loading control (n=4, ***P < 0.001 by unpaired 2 tail student’s t test).
4.2.7. The loss of *PDE4A* from the *ob/ob* mouse results in increased PDE4 activity at the membrane fraction of amygdala and in the cytosol fraction of hypothalamus.

PDE4 activity measurements from amygdala samples revealed a significant increase in the membrane of the *ob/ob:PDE4A*−/− compared to *ob/ob* mice (Fig.4.22), suggesting that the membrane fraction of amygdala and a PDE4 from a non-PDE4A subfamily might play role in this model of obesity.

![Graph showing PDE4 activity in amygdala](image)

**Figure 4.22.** Loss of the gene *PDE4A*−/− from the genetically obese mouse alters the levels of PDE4 activity at the membrane fraction of amygdala. PDE4 activity for the membrane or cytosol fraction of either *ob/ob* or *ob/ob:PDE4A*−/− mice on ND (n=7-10, **P < 0.01 by unpaired 2 tail student’s t-test)**
A significant increase in PDE4 activity was also observed in the cytosol fraction of hypothalamic samples of the \(ob/ob:PDE4A^{-/-}\) compared to \(ob/ob\) (Fig. 4.23), suggesting that the cAMP compartmentalization pathway and PDE4 localization of the genetic obesity induced depression phenotype might be slightly different than the dietary induced depression phenotype. Therefore further investigation on the differences between the two different obesity models is required.

Figure 4.23. Loss of PDE4A\(^{-/-}\) from the genetically obese mouse alters the levels of PDE4A activity at the cytosol fraction of hypothalamus. PDE4 activity for the membrane or cytosol fraction of either \(ob/ob\) or \(ob/ob:PDE4A^{-/-}\) mice on ND (n=8-11, ***\(P < 0.001\) by unpaired 2 tail student's t-test).
4.2.8. The loss of PDE4A gene impairs the rearing activity at the open field test

Open field measurements revealed no major differences in locomotor activity between WT and $PDE4A^{-/-}$ on both ND and HFD. Additionally, there were no significant differences from a direct comparison between the $ob/ob$ and $ob/ob:PDE4A^{-/-}$ (Fig. 4.24). The $ob/ob$ and $ob/ob:PDE4A^{-/-}$ mouse showed decreased locomotor and rearing activity at the open field test (Fig. 4.24) when compared with WT and PDE4A$^{-/-}$. Interestingly, the loss of the PDE4A gene from the WT and $ob/ob$ mice resulted in a decrease of the rearing activity measured at the open field test (Fig. 4.24). The reduced rearing activity that was observed after the loss of PDE4A gene was further reduced after the consumption of the HFD compared to ND (Fig. 4.24). The rearing activity represents the exploratory activity of mice. It has been suggested that decreased rearing activity might predict an autistic phenotype. Further investigation on the role of PDE4A in autistic behaviour is needed.

Figure 4.24. Genetic but not dietary obesity alters the total activity at the open field test. Open field test for PDE4A$^{+/+}$, PDE4A$^{-/-}$ on either ND and 3 weeks on HFD and $ob/ob$ and $ob/ob:PDE4A^{-/-}$ on ND (n=6-10, $P<0.05$, ***$P<0.001$, ****$P<0.0001$, by 1-WAY ANOVA with Tukey’s multiple comparison test).
4.2.9. The loss of PDE4A gene does not induce anxiety in the elevated plus maze test

Due to the causative role of anxiety to induce depression, anxiety related tests were performed for the WT, \textit{PDE4A}\textsuperscript{−/−} on HFD and \textit{ob/ob} and \textit{ob/ob:PDE4A}\textsuperscript{−/−} on ND to check for stress levels. The loss of PDE4A gene did not affect the anxiety behaviour measured in the elevated plus maze (EPM) test. Interestingly, in addition to the decreased total distance traversed in the closed arms, the \textit{ob/ob} and the \textit{ob/ob:PDE4A}\textsuperscript{−/−} mouse showed decrease entry to the closed arms (Fig. 4.25), suggesting an increased anxiety phenotype. This behaviour may be linked to the loss of leptin as both mice were missing the leptin gene. Therefore further tests should be done to explore this link.

Figure 4.25. Genetic but not dietary obesity alters the levels of anxiety at the elevated plus maze test. Elevated plus maze test for PDE4A\textsuperscript{−/−}, PDE4A\textsuperscript{−/−} on 3 weeks on HFD and \textit{ob/ob} and \textit{ob/ob:PDE4A}\textsuperscript{−/−} on ND (n=3-12*P<0.05, by 1-WAY ANOVA with Tukey’s multiple comparison post test).
4.2.10. Early dietary or genetic obesity does not induce gene expression changes for inflammatory genes in the hypothalamus

The role of different cytokines, especially TNF-α has been shown to be involved in brain dysfunction and disease (Clark et al., 2010) and it has been shown that cytokines can play a role in the development of depression (Raison et al., 2006; Schiepers et al., 2005). Clinical depression was suggested to arise due to cytokines released from activated macrophages (Smith, 1991). HFD has been shown to induce the tissue expression of TNF-α (Borst and Conover, 2005). To check whether inflammation is involved in this model of depression, expression analysis was performed for the main inflammatory cytokines in the hypothalamus. Real time PCR analysis on hypothalamic tissue samples from the WT, PDE4A<sup>−/−</sup>, ob/ob:PDE4A<sup>−/−</sup>, and ob/ob did not show any RNA expression differences of TNF-alpha (Fig. 4.26) or IL-1beta (Fig. 4.27).

**Figure 4.26.** No changes for the RNA levels of TNF-alpha after dietary or genetically induced obesity in the hypothalamus. Real-time PCR analysis of TNF-alpha for WT, PDE4A<sup>−/−</sup>, ob/ob and ob/ob:PDE4A<sup>−/−</sup> on either ND, 1 and 3 weeks on HFD (n=4).
Figure 4.27. No changes for the RNA levels of IL-1beta after dietary or genetically induced obesity in the hypothalamus. Real-time PCR analysis of IL-1beta for WT, PDE4A<sup>−/−</sup>, ob/ob and ob/ob:PDE4A<sup>−/−</sup> on either ND, 1 and 3 weeks on HFD (n=4)
However from the different cytokines, HFD increased the levels of IFN-γ only after the consumption of HFD for 3 weeks (Fig. 4.28 and Fig. 4.29).

Figure 4.28. No changes for the RNA levels of IFN-gamma after dietary or genetically induced obesity. Real-time PCR analysis of IFN-gamma for WT, PDE4A−/−, ob/ob and ob/ob:PDE4A−/− on either ND, 1 and 3 weeks on HFD (n=4).

Figure 4.29. HFD consumption induces an increase of IFN-gamma in the hypothalamus. Real-time PCR analysis of IFN-gamma for WT mice on ND and 3 weeks on HFD (n=4, **P < 0.01 by 2 tail unpaired Student’s t-test).
4.2.11. Dietary or genetic obesity does not alter BDNF RNA expression in the hypothalamus of mice

Neurotrophins have been shown to be involved in depression and in the mechanism of action of antidepressants (Pittenger and Duman, 2008) (Tardito et al., 2006) (Kozisek et al., 2008) (Chen et al., 2006) (Sakata et al., 2010). BDNF is a neurotrophic factor known for its antidepressant action via regulation of neurogenesis in the hippocampus (Schmidt and Duman, 2010). BDNF is also known to be regulated by cAMP levels (Conti et al., 2002). However, whether BDNF levels are altered in the hypothalamus in this model of depression is unknown. Real time PCR analysis on hypothalamic tissue from WT, \( PDE4A^{-/-} \), \( ob/ob: PDE4A^{-/-} \) and \( ob/ob \) mice revealed no significant differences in the expression levels of BDNF (Fig. 4.30).

**Figure 4.30.** No changes for the RNA levels of BDNF in the hypothalamus after dietary or genetically induced obesity. Real-time PCR analysis of BDNF for WT, \( PDE4A^{-/-} \), \( ob/ob \) and \( ob/ob: PDE4A^{-/-} \) on either ND, 1 and 3 weeks on HFD (n=4)
4.2.12. HFD slightly increases the total phosphorylation levels of DARPP32 in the hypothalamus of mice

DARPP32 is a downstream effector of the cAMP pathway phosphorylated by PKA (Helms et al., 2006). DARPP-32 plays an important role in schizophrenia (Cantrup et al., 2012). Consumption of HFD for 3 weeks slightly increased the total phosphorylation levels of DARPP32 (Fig. 4.31), suggesting that other molecular targets downstream of the PKA signalling pathway might play a role in obesity-induced depression phenotype. A phospho-proteomics study would be required to identify such targets.

Figure 4.31. High fat diet does not affect the total levels of p-DARPP32 in the hypothalamus. Western blot analysis for p-DARPP32 from mice fed ND versus 3 weeks on HFD. GAPDH was used as a loading control (n=4).
4.2.13. Loss of PDE4A in vivo does not affect body weight, food intake, glucose or insulin tolerance on HFD

Due to the role of HFD in the development of metabolic syndrome, a possible explanation for a lack of susceptibility to the depression phenotype exhibited by the PDE4A\(^{−/−}\) would be via resistance to the metabolic syndrome. Loss of PDE4A does not induce appetite or body weight changes on HFD (Fig. 4.32).

Figure 4.32. The loss of PDE4A does not induce any body weight changes or affect food intake on HFD. Food intake and body weight curves for PDE4A\(^{+/+}\) and PDE4A\(^{−/−}\) on HFD (n=5, by Linear mixed model fit by REML, Formula: weight ~ time + genotype + time:genotype + weight0 + (1 | id). Time (**P<0.001) and the interaction between time and drug (**P<0.01) have an effect on the body weight. The body weight at day 1 was used as a covariate and had no effect of the body weight gain).
In accordance, loss of PDE4A did not affect the insulin or glucose tolerance levels of mice measured after 9 weeks on HFD (Fig. 4.33). Finally, loss of PDE4A did not affect the weight of epididymal, inguinal fat, or liver, even after 9 weeks on HFD (Fig. 4.34).

**Figure 4.33.** The loss of PDE4A does not alter the glucose or insulin tolerance tests even after 9 weeks on HFD. Glucose tolerance and insulin tolerance test for PDE4A<sup>+/+</sup> and PDE4A<sup>−/−</sup> on 9 weeks on HFD (n=5). This work was performed with Dr. Bernat Baeza Raja.

**Figure 4.34.** The loss of PDE4A does not alter neither the white adipose tissue size nor the liver even after 9 weeks on HFD. Total weight of epididymal fat (IP), inguinal fat and liver for PDE4A<sup>+/+</sup> and PDE4A<sup>−/−</sup> on 9 weeks on HFD (n=5).
4.2.14. PDE4A5 overexpression induces an increase in body weight

To investigate the role of PDE4A5 specifically in the hypothalamus, an adeno-associated virus (AAV) overexpression system was employed. The overexpression of PDE4A5 was performed in PDE4A\(\beta/\beta\) mice, therefore overexpression of the PDE4A5 in the hypothalamus will reveal the specific role for this enzyme in this brain region. A map of the adeno-associated virus (AAV) construct that overexpresses PDE4A5-vsv under the synapsin promoter is shown in Fig. 2.2. A map of the control AAV used in this study overexpressing eGFP is shown in Fig. 2.1. As mentioned above, PDE4A\(\beta/\beta\) mice are resistant to the dietary or genetic obesity-induced depression phenotype (Fig. 4.16). Therefore, we wanted to test whether overexpression of PDE4A5 specifically in the hypothalamic area of this KO mouse would induce a depression phenotype. Bilateral stereotactic injections of the virus overexpressing PDE4A5 or eGFP in the hypothalamic area of PDE4A\(\beta/\beta\) mice were conducted. Immediately after injection, animals were provided a HFD for 21 days. After the completion of the 3 weeks on HFD, behavioural tests were performed to assess whether overexpression of PDE4A5 in the hypothalamus is sufficient to induce a depression phenotype.
Surprisingly, following exposure to HFD, bilateral infection of the hypothalamic areas of $PDE4A^{-/-}$ mice with the AAV-PDE4A5wt-VSV induced extreme body weight gain compared to the mice that received bilateral injections of the control AAV-GFP (Fig. 4.35).

Figure 4.35. Overexpression of PDE4A5 in the hypothalamic area of PDE4A^{-/-} mice results in an increase in body weight. Body weight measurements of PDE4A^{-/-} mice that received either AAV-Synapsin-PDE4A5vsv or AAV-Synapsin-eGFP and where on HFD for 3 weeks (n=4 unpaired two tail student’s t-test ***P<0.001).
The immobilization time for the AAV-PDE4A5wt-VSV was reduced but not significantly different to the control injected mice (Fig. 4.38). Because of the excess body weight gain, no conclusions could be made about the role of PDE4A5 in development of the depression phenotype as the massive increase in body weight can play a role in the development of depression in mice. Even though previously it was shown that body weight gain might not affect the performance to the different behavioural tests the extreme body weight gain that the mice exhibited after the virus injections might have an effect as it can not be compared with the previous body weights.

Figure 4.36. Overexpression of PDE4A5 in the hypothalamic area of PDE4A\(^{-/-}\) mice does not affect the immobility after HFD. Immobilization time of PDE4A\(^{-/-}\) mice that received either AAV-Synapsin-PDE4A5vsv or AAV-Synapsin-eGFP and were on HFD for 3 weeks (n=4)
After the completion of behavioural tests, western blot analysis was performed in order to confirm the exogenous expression of the virus. Expression analysis revealed high levels of the vsv protein in the hypothalamus (Fig. 4.39). AAV-mediated EGFP expression was restricted to the deposition site of the hypothalamic area indicating very limited diffusion of AAV vector in the brain as there was no detection in amygdala, which is a neighbouring area (Fig. 4.39).

Figure 4.37. AAV mediated overexpression of PDE4A5 and GFP in the hypothalamous and amygdala. Western blot analysis for vsv and GFP for the PDE4A⁻/⁻ mice injected with either the AAV-PDE4A5vsv or AAV-eGFP.
4.3. Discussion

One possible target of antidepressant drug administration is the cAMP signal transduction system (Duman et al., 1997). Chronic, but not acute, antidepressant treatment up-regulates this intracellular cascade at several levels (Nestler et al., 1989b; Ozawa and Rasenick, 1991; Nibuya et al., 1996b; Duman et al., 1997; Perez et al., 1989). Major modulators of the cAMP signalling cascade are the phosphodiesterase enzymes, which serve to hydrolyse the cAMP to 5’AMP and terminate its signal transduction. The driving notion behind this present study is that PDEs contribute to the generation of intracellular cAMP microdomains, which are critical for the link between obesity and depression. Phosphodiesterases are localized in areas of the brain that are associated with reinforcement, movement, and affect all of which are impaired in people with depression (Cherry and Davis, 1999). There is a lot of epidemiological evidence to support the high prevalence of depression observed in overweight and obese individuals (Zhong et al., 2010; Leonore de Wit and Frans Zitman, 2010; Boutelle et al., 2010; Stunkard et al., 2003; Simon et al., 2010; McElroy et al., 2004; Faith et al., 2002; Luppino et al., 2010; Palinkas et al., 1996; Roberts et al., 2003; Simon et al., 2010; Dong et al., 2004; Roberts et al., 2002). It is also worth noting that overweight and obese individuals exhibit resistance to antidepressant treatment (Papakostas et al., 2005). However, little is known about the exact molecular mechanism that link obesity with depression and whether phosphodiesterases play a role on this pathway. With these statements in mind, I wanted to determine whether phosphodiesterases are involved in the pathway that links obesity with depression. I hypothesized that identification of the specific phosphodiesterase isoform(s) involved would shed light on the signalling mechanisms which underpin this link and may help the development of more specific therapeutic strategies to target this specific type of depression.
4.3.1. The role of rolipram in the dietary obesity induced depression phenotype

Rolipram is a small molecule with multiple \textit{in vivo} functions, for example, as an antidepressant and an anti-inflammatory (Zhu et al., 2001; O’Donnell and Zhang, 2004). Its actions stem from its ability to inhibit all isoforms from the PDE4 family, the major regulators of the cAMP signalling in a wide variety of cells. In this study it was found that chronic but not acute rolipram administration can partially inhibit the dietary obesity-induced depression phenotype that was observed in C57Bl/6J mice. This is in accordance with previous studies that proposed that the necessary changes that underlie rolipram’s antidepressant effect are chronic and not acute and are required for their beneficial clinical efficacy in depressed patients (Tardito et al., 2006; Przegalinski et al., 1985).

HFD induces obesity, the metabolic syndrome (Hwang et al., 2010), as well as high expression levels of TNF-\(\alpha\), a pro-inflammatory cytokine (Borst and Conover, 2005). On the other hand, obesity is a disorder that is associated with an upregulation of the inflammatory response system (IRS) where the main characteristic is the hyperproduction of inflammatory cytokines (Makki et al., 2013) that associates with the development of brain disease (Makki et al., 2013) and depression (Schiepers et al., 2005). Low-grade inflammation is frequently present among patients with symptoms of depression (Bremmer et al., 2008; Gimeno et al., 2009). Antidepressant treatment also affects the levels of different cytokines (Kenis and Maes, 2002). A possible explanation for the resistance to depression phenotype after rolipram treatment is the anti-inflammatory effects of rolipram, which downregulates cytokine expression and has been proposed for the treatment of inflammatory diseases (Houslay et al., 2005). Rolipram suppresses the production of cytokines such as TNF-\(\alpha\), lymphotoxin-\(\alpha\) and IFN-\(\gamma\), which lead to the suppression of experimental autoimmune encephalomyelitis, an inflammatory disease (Sommer et al., 1995). The cytokine TNF-alpha is a necessary element in the chain of pathophysiologic events leading to inflammation. The PDE4 isoforms are highly expressed in monocytes, which are a main source of TNF production, and rolipram is a 500-fold more potent inhibitor of TNF synthesis in human mononuclear cells compared with the nonspecific PDE inhibitor pentoxifylline (Semmler et al., 1993).
However, real-time PCR analysis in my study (Fig. 4.26, 4.27, 4.28) revealed no changes in levels of cytokines in the hypothalamus of WT and PDE4A<sup>−/−</sup> mice fed either ND or HFD. Measurements of the circulating cytokines in the periphery or in other brain regions, though, are necessary to dissect their exact role in this model. Although we were not able to detect changes in cytokines, one cannot exclude the possibility that durable modifications were caused by transient, but sizable elevations of cytokines at an earlier stage that we did not study.

One can also take into consideration that HFD has been shown to affect the binding of rolipram to phosphodiesterase 4 in the hypothalamus (Greene et al., 2009b). Acute treatment with desipramine increases the norepinephrine release as well as the total binding of rolipram in the hypothalamus (Greene et al., 2009b). By contrast, rats that were fed a HFD did not respond to the acute desipramine treatment, and revealed less binding of rolipram in the hypothalamus compared to ND fed rats (Greene et al., 2009b). The data in the previous study suggests that rolipram injections in our model might be affected by the consumption of the HFD, therefore further investigation should be done to determine the interaction between rolipram and HFD consumption.

Another possible explanation for the resistance to depression phenotype of the mice that received rolipram could be deduced from the role of diabetes in depression. Consumption of 60% fat induces diabetic characteristics in mice (Hwang et al., 2010). Rolipram has been shown to prevent diabetes in a mouse model of insulitis by blocking the production of inflammatory cytokines such as IL-12, IFN-γ, TNF-α in macrophages (Liang, Beshay et al. 1998). However the role of diabetes in my model can be excluded by the GTT and ITT measurements from the PDE4A<sup>−/−</sup> and their litter mate controls PDE4A<sup>+/+</sup> mice (Fig. 4.33). Even 9 weeks on HFD failed to induce differences in glucose or insulin tolerance levels suggesting that both groups have risk of diabetes even though the PDE4A<sup>−/−</sup> showed a resistance towards the obesity induced depression phenotype. Moreover, in the current study, behavioural assays were performed on animals that were on HFD for 3 weeks, which is not enough time for the development of the complete characteristics of glucose and insulin resistance shown in diabetes.
Another possible explanation for the resistance to depression phenotype exhibited by the mice that received rolipram is the role of the hypothalamic pituitary axis (HPA). The HPA pathway is an important signalling axis that connects cytokine production with hypothalamic activity (Turnbull and Rivier, 1995). Rolipram has been shown to stimulate the HPA axis by acting at the levels of both the hypothalamus and the pituitary gland (Kumari, Cover et al. 1997). The activation and the role of the HPA pathway in the dietary-induced depression phenotype should be further studied.

4.3.2. The effect of rolipram in the dietary-induced obesity phenotype

In this study, daily injections of rolipram to C57Bl/6J mice resulted in resistance to the dietary-induced obesity phenotype that is normally seen in this strain. This is in accordance with other recent work that reported rolipram’s anti-obesity effects (Park et al., 2012; Doseyici et al., 2014; Chung, 2012). In accordance, clinical trials with roflumilast, another PDE4 inhibitor that is used for the treatment of COPD revealed a weight loss for the patients treated with roflumilast compare to placebo (Calverley et al., 2009; Rabe, 2011). Cytokine production has been involved in the development of obesity induced by HFD consumption (Borst and Conover, 2005). Since rolipram blocks cytokine production (Liang, Beshay et al. 1998), it might affect the development of dietary-induced obesity as well. The PDE4 isoform responsible for the anti-obesity effect of rolipram is unknown. However, it has already been published that loss of PDE4B suppresses TNF-α mRNA levels and PDE4B−/− mice are leaner compare to their litter-mates after the consumption of a HFD (Zhang et al., 2009). In particular, these mice produce smaller fat pads, an indication that suggests resistance to dietary-induced obesity (Zhang et al., 2009). Such a result points to the fact that PDE4B is a good candidate to target in order to suppress obesity. If this is true, one could extrapolate these findings to the current study to propose that rolipram administration inhibited the PDE4B sub-family in fat tissue and this mechanism might be responsible for the resistance to the diet-induced obesity. Specific inhibition of the PDE4B in fat or other tissues might
shed light whether PDE4B is the enzyme responsible for the resistance to the dietary-induced obesity phenotype.

Finally, even though in this model body weight gain does not affect performance in the depression-related tests (Chapter 3, Fig. 3.5), there is always a possibility that rolipram-injected mice are partially rescued from dietary-induced depression phenotype due to the fact that they did not gain weight. However, this hypothesis is not supported as both Prozac- and rolipram-injected mice showed an antidepressant phenotype compared to the saline-injected mice. Prozac-injected mice gained significantly more weight and the rolipram-injected did not gain any weight, further supporting the lack of correlation between body weight and depression phenotype. Recently the clinical trials for rionabant (CB1R antagonist) as an antiobesity and anorectic drug was suspended because of increased risks of serious psychiatric problems, including depression (Samat et al., 2008). Therefore, finding the specific isoform of the PDE4 family responsible for the antiobesity effect of rolipram can be used as a potential target for obesity without the psychiatric side effect of depression.

4.3.3. The role of Prozac in the dietary obesity induced depression phenotype

Prozac is a popular, widely prescribed antidepressant and like most of the other SSRIs, it has been linked with body weight gain (Schwartz et al., 2004). However, whether this body weight gain is a direct effect of Prozac or a secondary effect due to euphoria that the depressed patients experience under antidepressant treatment remains unknown. Moreover, even though it has been shown to improve depression symptoms, the effects of Prozac in other tissues have not been investigated. In this study, I have reported that Prozac-injected mice gained significantly more weight compared to the saline-injected mice while they were on HFD. This is an early effect occurring directly after the first Prozac injection (Fig. 4.4), suggesting a direct effect of Prozac in body weight. This data should provoke thoughts on whether Prozac should be further prescribed due to its side effect on body weight gain. Moreover, this study showed that daily Prozac injections led to liver enlargement, compared to saline-injected mice.
In agreement with my observations, the effect of Prozac in liver induces lipid accumulation via both the SREBP1c-related lipogenesis and reduction of lipolysis in primary mouse hepatocytes (Feng et al., 2012). Further analysis of the molecular action of Prozac in liver after HFD, as well as the role of Prozac on ND is necessary to dissect whether this effect is specific for mice on a HFD. Due to the side effects of Prozac administration in this study, I consider rolipram to be a better antidepressant drug (in comparison to Prozac), as it did not induce body weight gain or liver enlargement.

In contrast to the results in this study, it has been described that Prozac, suppresses appetite in animals (Feldman and Smith, 1978; Kim and Wurtman, 1988; Li et al., 1996; Luo and Li, 1990) and promotes weight loss in humans (Goldstein et al., 1994; Goldstein et al., 1995; Levine et al., 1987; Levine et al., 1989). These studies suggest that serotonin has a suppressive effect on food intake and body weight and it is implicated in the regulation of feeding (Grignaschi and Samanin, 1992). Central administration of serotonin to ob/ob and WT mice induced a reduction in food intake in both groups (Currie and Wilson, 1992). The ob/ob mice showed an impaired sensitivity to serotonin in relation to food intake compare to control mice and this might be the result from impaired satiety mechanism in this mouse model of obesity (Currie and Wilson, 1992). However, chronic administration of Prozac failed to induce any changes in the body weight or food intake of the ob/ob mice (Dubuc and Peterson, 1990). Stimulation of 5-HT\textsubscript{1A} receptor results in hyperphagia whereas 5-HT\textsubscript{1B} receptor stimulation mediate hypophagia in rats (Curzon, 1990; Dourish et al., 1986; Hutson et al., 1988; Kennett and Curzon, 1988; Weiss et al., 1986). Mice lacking functional 5-HT\textsubscript{2C} receptors are hyperphagic and become obese (Nonogaki et al., 1998) suggesting that different serotonin receptors have a different effect. Therefore, it is believed that combination of fluoxetine with 5-HT\textsubscript{1A} receptor antagonist could be of clinical utility in the treatment of eating disorders and obesity (Li et al., 1998).

According to the previous studies, we would expect that mice that received Prozac would not gain as much weight as the saline-injected mice. However, they gained significantly more weight even from the first injection. The data in
our study suggest that the consumption of HFD interferes with the activity of serotonin receptors, as the previous studies were performed on ND. It has already been suggested that fatty acids can interfere with the serotonergic receptor signalling (Kodas et al., 2004), where fatty acids can have a differential effect (du Bois et al., 2006). Therefore, further investigation on the interaction between serotonin and the consumption of dietary patterns rich in specific fatty acids should be studied. Furthermore, even though serotonin has been shown to inhibit appetite (Currie and Wilson, 1992), further investigation in this model whether Prozac administration increased appetite of mice on HFD should be undertaken, as this could be the underlying cause of increased body weight gain.

In this study, daily i.p. injections of rolipram or Prozac were performed in order to investigate their role in the onset of complications caused by HFD. However, repeated injections are expected to cause peaks and troughs in plasma concentration, which would be more extreme in animals with more rapid metabolism. In addition, repeated i.p. injections are clearly stressful to the animals and it is possible that the antidepressant effect induced by daily rolipram or Prozac injections may only occur in the presence of stress. A better way to answer this question would be the implantation of an osmotic pump which will produce relatively stable plasma levels. In agreement with this notion, it has been reported that PDE4 can interact with different partners in the *in vivo milieu* due to different stimulators such as stress, and this can affect the affinity for rolipram for PDE4 enzymes (Houslay and Adams, 2003).

### 4.3.4. The role of the phosphodiesterase 4A subfamily in the diet induced depression phenotype

cAMP plays a prominent role in cellular signalling pathways in neurons and processes of neuroplasticity and possibly has a critical role in brain disease (Kandel, 2012). Phosphodiesterase-4A (PDE4A) is one of the four genes encoded by the PDE4 family which enzymatically regulate spatial and temporal cAMP signalling throughout the mammalian organism (Conti et al., 1992; Houslay 2001). PDE4 enzymes are known for their lack of modulation of cGMP (is cAMP specific) and its sensitivity to the antidepressant drug rolipram (Beavo et al., 1994; Bolger 1994; Beavo 1995).
In this study, I have shown for the first time that HFD results in the isoform specific upregulation of RNA and protein levels of the PDE4A5 in the hypothalamus of WT mice (Fig.4.13&4.14). Interestingly, the phosphorylation state and hence activity of the PDE4A5 is also enhanced by HFD (Fig.4.21).

Antidepressant treatment alters PDE4 activity (Zhao et al., 2003) and the levels of PDE4A in the brain (Takahashi et al., 1999; Ye et al., 2000; D'Sa et al., 2005; Dlaboga et al., 2006; Fujita et al., 2007; Fatemi et al., 2010). Studies in rats have shown an increase in PDE4A expression in frontal cortex (Takahashi et al., 1999; D'Sa et al., 2005), hippocampus (Ye Y, 2000; Miro et al., 2002), parietal cortex (D'Sa et al., 2005) and cerebral cortex (Ye et al., 1997; Zhang et al., 1999) as a result of chronic antidepressant treatment. Upregulation of PDE4 is not limited to antidepressant drug treatment, as chronic electroconvulsive seizure also increases PDE4 activity and enhances PDE4 transcription (Takahashi et al., 1999; Suda et al., 1998). On the contrary to previous studies that revealed PDE4A upregulation, it has been shown that specific PDE4A isoforms were significantly decreased in the cerebellum of patients with bipolar disorder (Fatemi et al., 2008) and there was no change in patients with schizophrenia or major depression (Fatemi et al., 2008), suggesting the importance of the localization for the specific targeting of the cAMP degradation to the different psychiatric disorders. This is further proven by the distinct pattern of PDE4A gene family expression, suggesting their different functional roles in the brain (McPhee et al., 2001). Taking all of these observations together, increased PDE4A5 after consumption of the HFD - that is proposed to lead to the depression phenotype - is in accordance with the increased PDE4A upregulation after antidepressant treatment. This is as a result of a compensatory mechanism that can inhibit the antidepressant effect caused by antidepressants (Duman et al., 1997).

Although rolipram has been proven to be an effective antidepressant in animal studies, it failed in clinical trials due to serious side effects such as nausea and emesis that were induced in humans. High levels of PDE4D are found in brain regions known to be involved in emesis and nausea (Cherry and Davis, 1999;
Miller and Leslie, 1994; Robichaud et al., 2002). It has been suggested that inhibition of PDE4D in these areas induces emesis via shortening of a2-adrenoceptor-mediated anesthesia, a behaviour that correlates with emesis. PDE4A is expressed relatively low in areas of the brain that are involved in emesis compare to PDE4D that is highly expressed. As shown in this study, the PDE4A5 isoform is responsible for the obesity induced depression phenotype. Therefore, it is possible that increased expression and activity of PDE4A5 could reduce the maximal response to antidepressant treatment. Hence, specific inhibition of this enzyme would be a promising therapeutic target, as it does not carry the side effect of emesis. Combination of a specific PDE4A5 inhibitor could enhance the response to other classes of antidepressants but without the side effects of nonselective inhibitors. An effective PDE4A specific inhibitor has yet to be discovered. A pair of conserved histidines on the PDE4A has been shown to play important role in the activity of the enzyme (Omburo et al., 1998) that might help the discovery of a specific inhibitor.

4.3.5. Post-translational modifications of the PDE4As might play a role in the dietary obesity induced depression phenotype

The activity of long PDE4s isoform is regulated by phosphorylation within a PKA consensus motif located within the UCR1 region (Conti et al., 1995) and studies using recombinant PDE4s showed that the phosphorylation also alters the binding affinity of rolipram (Hoffmann et al., 1998; Sette and Conti, 1996). In this study we showed increased phosphorylation of PDE4A5 by PKA (Fig.4.21), and this is suggested to increase the PDE4 activity observed after HFD (Fig.4.7).

Phosphorylation of PDE4A5 by MK2 (MAPKAPK2) attenuates its activation through protein kinase A phosphorylation, and selectively disrupts its ability to be sequestered by those partner proteins whose interaction involves UCR2, as it induces a structural change (MacKenzie et al., 2011). MK2 is a downstream effector of p38 MAPK, which is a signalling cascade and a key signal transduction pathway involved in the control of cellular immune, inflammatory and stress responses (Cowan and Storey 2003). Therefore, increased inflammation after HFD might play a role in the regulation of the PDE4A activity in my model.
Further investigation into MK2 kinase activity in the hypothalamus after HFD is needed to assess the role of the p38 MAPK pathway. Work using a phospho-specific antibody to the MK2 site on PDE4A5 (MacKenzie et al., 2011) is also required to substantiate the role of this phospho-site in the link between obesity and depression.

### 4.3.6. Compartmentalization of cAMP signalling underpinned by PDE4A activity

PDE4A is widely expressed throughout the rodent brain, but regional and intracellular distribution for each subtype is distinct (McPhee et al., 2001). This differential distribution suggests that the PDE4A subtypes may serve different functions, as it is known that individual PDE4 isoforms have unique non-redundant roles conferred by their cellular location. The data in this study lends support to this notion, as abolishment of PDE4 activity at the membrane fraction of the $PDE4A^{-/-}$ mice (after HFD) rescued the increase of the PDE4 activity observed for the WT mice after HFD that is believed to cause depression. This further shows that diet specifically affects the localization of enzymatic activity from the PDE4A subfamily. It is also noteworthy that there is an absence of any compensatory mechanism from other phosphodiesterases. Moreover, HFD increases the phosphorylation and therefore activity levels of PDE4A5 (Fig.4.21 & 4.21). However, HFD was shown to decrease the PKA signalling which should result in less PKA phosphorylation of its substrates (Fig.3.11). There are two possible explanations for that controversy. The first one is that PKA is localized in close proximity with PDE4A5 so that PDE4A5 is rapidly phosphorylated when PKA is active. The second explanation might be that PKA has a high affinity for PDE4A5 therefore even in low levels it can still phosphorylate and activate PDE4A5. Both explanations support the idea of the compartmentalization and temporal and special regulation of the cAMP signalling.

There is increasing evidence to indicate that the inhibition of these PDE4As activities is associated with depression, as they are activated as a compensatory mechanism to downregulate cAMP signalling. In support of the data in this study, repeated antidepressant treatment selectively increases the PDE4 activity at the membrane fractions of hippocampus and cerebral cortex (Zhao et al., 2003). The
increased PDE4 at the membrane fraction may be a compensatory response to regulate intracellular cAMP concentrations and may represent another component of cAMP mediated signal transduction systems affected by repeated administration of antidepressants from different pharmacological classes. That increase of the PDE4 activity at the membrane fraction shown in this study can happen either by increased protein expression or post-translational modification. The interaction of the PDE4s with other cellular proteins has also been suggested to be involved.

4.3.7. HFD does not induce an anxiety phenotype in mice

It is generally accepted that repeated stressful events can induce depression in humans and mice (Nestler et al., 2002). Therefore, to exclude stress being the triggering factor for depression, we examined the anxiety levels of the mice using the EPM. No significant differences were observed after HFD (Fig. 4.25), which indicates that our depression phenotype was independent of the anxiety state of the mice. Therefore, in our model of depression, we were able to isolate and measure the depression phenotype independently of the development of anxiety phenotype. Moreover, it has been shown that loss of PDE4B can be responsible for the anxiogenic effect in mice, further underscoring the specific roles of different PDE4 isoforms in brain (Zhang et al., 2008) and suggesting that the inhibition of PDE4A will not increase any anxiety behaviour.

4.3.8. PDE4A is responsible for the rearing activity in mice

The loss of PDE4A gene did not induce any anxiety related behaviours as measured using the EPM. However, it reduced the rearing activity of mice on ND, and this deteriorated after HFD. Rearing activity is a measure of the exploratory activity in mice and it has been implicated in autism disorders. In agreement with this fact, reduced PDE4A5 levels have been discovered in the cerebella of patients with autism (Braun et al., 2007). Further investigation on the link between autism and a reduction in PDE4A activity is needed. Although these mice have not been characterized, an observation made in this study was the extreme grooming of their face, another indication of autism.
4.3.9. The role of CREB in obesity-induced depression phenotype

Many signaling pathways involving 2nd messengers such as cAMP and calcium lead to the phosphorylation of CREB at serine-119. This event allows the recruitment of the essential coactivator CBP to CREB (Shaywitz and Greenberg, 1999). However, multiple signalling pathways are known to regulate CREB activity at different levels and those that depend on the calcium/calmodulin dependent protein phosphatase calcineurin, control the transcriptional activity of CBP after its recruitment by phospho-CREB (Oetjen et al., 2005; Oetjen et al., 2006; Schwaninger et al., 1995a; Schwaninger et al., 1995b) or govern the nuclear translocation and binding to the CREB leucine zipper domain of another essential CREB coactivator, TORC (Conkright et al., 2003; Ravnskjaer et al., 2007).

There is much controversy concerning the role of CREB in depression (Carlezon et al., 2005) and the actions of CREB may not be as uniform as to fit into a simple pro-depressive/anti-depressive scheme. Rather, the outcome of CREB action may depend on the duration of its action, the level of activation, the specific brain region, and the particular environmental conditions involved in the experiment. Moreover, it is also possible that chronic antidepressant treatment regulates the expression or function of other transcription factors in addition to CREB (Nibuya et al., 1996a) or that CREB leads to regulation of other target genes, which may also play a role in the action of antidepressant treatments. Promoter analysis of differentially expressed genes in depression revealed a clear enrichment of binding sites for the transcription factor CREB1, which is a molecule involved in epigenetic regulation (cAMP response element-binding protein induces histone modifications) (Lagus et al., 2010). It is noteworthy that CREB1 might also constitute one of the major links between disturbed sleep habits and mood (Lagus et al., 2010), both important characteristics impaired in depression.

In this study, downregulation of the PKA-mediated phospho-CREB levels was observed after the consumption of HFD (Fig. 4.15). This is in accordance with previous expression data in this study that showed that the PKA signalling pathway was significantly suppressed in the hypothalamus after HFD (Chapter 3,
CREB is an important transcriptional factor regulating a variety of pathways and important genes such as BDNF. Further understanding of the exact role of CREB in our model should be facilitated via the use of inducible hypothalamus-specific CREB transgenic mouse, which has already been used to investigate the role of CREB after treatment with psychostimulants (Sakai et al., 2002). There are different effects on CREB from antidepressant drugs compared with non-antidepressant psychotrophic drugs and it has been shown that only chronic antidepressant treatment increases the expression of CREB in rat hippocampus (Nibuya et al., 1996a). Increased expression of CREB was observed after chronic but not acute antidepressant treatment demonstrating that upregulation of CREB is dependent on repeated antidepressant treatment (Nibuya et al., 1996a) and this correlated with increased levels of BDNF and TrkB in the hippocampus (Nibuya et al., 1995). Taken together, these studies indicate that increased expression of CREB and induction of BDNF and TrkB in response to chronic antidepressant treatments could increase the survival and/or function of neurons as this has been reported for direct application of BDNF (Ghosh et al., 1994; Lindsay et al., 1994; Lindvall et al., 1994).

Sustained elevation of cAMP via chronic administration of rolipram increases the expression and phosphorylation of CREB, as well as BDNF and TrkB, and facilitates the action of antidepressants on expression of these proteins (Nibuya et al., 1996a). Even though all these effects have been proposed for the function of CREB in the hippocampus, a similar model might explain the development of depression phenotype in the hypothalamus after the consumption of a HFD. Reduced phosphorylation of CREB in the hypothalamus might lead to decreased transcriptional activation of genes regulated by CREB and this transcriptional regulation might lead to the development of depression. Further investigation on the role of CREB in a cell-type and brain region specific in the hypothalamus is needed to further characterize its role on this pathway.
It has already been shown that coadministration of rolipram with an antidepressant drug resulted in a more rapid induction of CREB than with either treatment alone (Nibuya et al., 1996a). Therefore the identification of the isoform responsible for the obesity induced depression phenotype in this study and the creation of a specific inhibitor targeting PDE4A5 might be able to be used in combination with current antidepressants and have a faster effect. Clinical studies are required to determine whether coadministration of a PDE inhibitor is capable of shortening the time required for the therapeutic action of antidepressant treatments. This might be most beneficial for people with severe forms of depression and antidepressant effect is urgently needed.

4.3.10. Neurotrophins and their role in depression

Infusions of BDNF into the dorsal raphe nucleus produce an antidepressant effect, as evaluated by several ‘learned helplessness’ paradigms (Siuciak et al., 1997). Chronic but not acute antidepressant treatment of rats elevated the BDNF and TrkB RNA in the rat hippocampus (Fujimaki et al., 2000; Nibuya et al., 1995; Duman et al., 1997) suggesting that many antidepressants act via the upregulation of neurotrophins. Further evidence that BDNF and depression are linked together has been gleaned from reports that describe stress acting as an inducer of depression that results in downregulation of the RNA levels of BDNF. Treatment with psychopharmacological drugs such as cocaine did not affect the levels of BDNF or TrkB suggesting the specificity of the neurotrophins for the depression pathway (Nibuya et al., 1995). Induction of seizures in rats by pilocarpine (Schmidt-Kastner et al., 1996) or by electroshock (Nibuya et al., 1995; Smith et al., 1997) which mimics the ECT used to treat severe depression, also elevated BDNF RNA in the hippocampus, cortex and other brain regions. It has already been shown that BDNF in the hippocampus of mice fed HFD was significantly lower than mice on ND mice (Yamada et al., 2011), and leptin administration was able to increase hippocampal BDNF in the mice fed normal diet compare to HFD (Yamada et al., 2011). A major target of the phosphorylated CREB is BDNF transcriptional regulation. However even though we detected a downregulation of the phosphorylation of CREB, the present study failed to demonstrate any RNA changes of BDNF in the hypothalamus after HFD, suggesting that another pathway might be involved in the hypothalamus.
4.3.11. Virus mediated overexpression of PDE4A5 in the hypothalamus induces hyperphagia and obesity

The data in this study provide strong evidence that AAV-mediated overexpression of PDE4A5 in the hypothalamic area can promote obesity possibly via hyperphagia in response to the dietary challenge in the adult mice. This implicates the PDE4A5 and cAMP signalling in eating disorders. The strikingly body weight increase of the PDE4Aβ/β mice after the overexpression of PDE4A5-wt in the hypothalamic area was an unexpected result.

The hypothalamic melanocortin system is a predominant pathway in appetite regulation and energy homeostasis (Adan et al., 2006; Seeley et al., 2004; Vergoni and Bertolini, 2000). The melanocortin-4 receptor (MC4R) in the central nervous system (CNS) plays a critical role in the control of energy balance. Activation of MC4R by α-Melanocytewstimulating hormone (α-MSH), a derivative of proopiomelanocortin (POMC), decreases food intake and increases energy expenditure. Conversely, inhibition of MC4R by agouti-related protein (AgRP) increases food intake and decreases energy expenditure. MC4R deficiency in mice or humans results in hyperphagia and a severe obesity syndrome (Huszar et al., 1997; Yeo et al., 1998; Vaisse et al., 2000). MC4R is broadly expressed in many brain areas implicated in the regulation of energy balance (Mountjoy et al., 1994; Kim et al., 2000; Kishi et al., 2003; Lu et al., 2003; Kas et al., 2004) and is highly expressed in the paraventricular nucleus (PVN) of hypothalamus (Mountjoy et al., 1994; Kishi et al., 2003; Lu et al., 2003). Studies suggest that the PVN is one of the key neuroanatomical regions in the brain in mediating MC4R signaling and the regulation of energy homeostasis. Lesions of the PVN result in hyperphagia in rats and mice (Weingarten et al., 1985; Sawchenko 1988; Sawchenko 1998). Microinjection of MC4R agonist (MTII or α-MSH) into the PVN inhibits food intake, whereas microinjection of MC4R antagonists AgRP, SHU9119 or HS014 in the PVN stimulates food intake (Cowley et al., 1999; Kask et al., 2000; Kim et al., 2000; Wirth and Giraudo 2001). It has also been demonstrated that overexpression of MC4R antagonist agouti in the PVN using AAV causes increased food intake and body weight (Kas et al., 2004). Interestingly, adeno-associated virus-mediated knockdown of melanocortin-4...
receptor in the paraventricular nucleus of the hypothalamus promotes high-fat diet-induced hyperphagia and obesity (Garza et al., 2008). These data support the importance of MC4R activity in the PVN in the control of appetite and body weight. The intracellular downstream pathway of the MC4R is unknown and should be investigated.

After consideration of the data resulting from my study, the cAMP signalling cascade might play an important role in eating disorders and regulating appetite. Further investigation whether PDE4A5 is a major modulator of appetite is needed as it could be considered as a novel target for appetite disorders.

A possible caveat in this study is the fact that overexpression of PDE4A5 was done in the PDE4Aβ/β mice, therefore whether the effect is specific for the PDE4Aβ/β mice or happens in the WT mice is unknown. Further investigation of whether the major driver of the development of obesity is the hyperphagia or whether there is a fatty acid oxidation defect or a defect with dysregulation of the fatty tissue is warranted. Another possible explanation is that by only introducing the PDE4A5 enzyme in the hypothalamic area of the PDE4Aβ/β mice we disturbed a fine balance of cAMP homeostasis that existed in the PDE4Aβ/β mice, and perhaps that is the reason why we failed to notice this phenotype in the PDE4Aβ/β. Further studies overexpressing other PDE4 isoforms are needed to test for the specificity of the PDE4A5 involved in obesity.

AMP-activated protein kinase (AMPK) plays a major role in regulating energy homeostasis and feeding in the central nervous system (Ruderman et al., 2003) (Andersson et al., 2004), and is activated by metabolic stress such as nutrient starvation (da Silva Xavier et al., 2000). AMPK is phosphorylated and activated when the energy levels are low. Further investigation of the role of AMPK and a possible link with the cAMP pathway should be done. Further studies should also count weekly the food intake and the body weight.
4.3.12. Different brain regions might be involved in the obesity induced depression phenotype

This study focuses on the role of the hypothalamus in the obesity-induced depression phenotype. Hypothalamus is a brain area that has historically been known to be involved in both obesity and depression. The hypothalamus is regarded as the main centre for regulating appetite and obesity has been linked with eating disorders (Ryan et al., 2012; Schwartz et al., 2000; Woods and D’Alessio, 2008). Using positron emission tomography, a disruption of neural circuits linking the cortex, thalamus and cerebellum (cortico-thalamic-cerebellar-cortical circuit) has been described in patients with schizophrenia (Andreasen et al., 1996). Functional imaging studies of patients with depression show reduced cerebellar activation (Beauregard et al., 1998; Liotti et al., 2002) which persisted even in patients that had recovered from depression (Smith et al., 2002). This effect did not correlate with the changes in β-adrenergic receptor densities, suggesting antidepressants alter intracellular signal transduction pathways in a similar manner (Ye et al., 2000). Further investigation of the other brain regions connected to the hypothalamus is needed in order to determine the role of the other brain regions in the obesity induced depression phenotype.

Studies using autoradiography that detected changes of PDE4 mRNA expression in small areas of the brain, such as subregions of hippocampus and selected thalamic nuclei after antidepressant treatment (D’Sa et al., 2005; Miro et al., 2002), suggest the specificity of the compartmentalization signalling by PDE4s in specific and defined brain regions. This should be supported by the development of mouse models exhibiting brain region specific overexpression and deletion of individual PDE4 isoforms. The differential distribution of the different PDE4A isoforms indicates the distinct roles of individual isoforms in CNS function further indicating that the development of isoform-specific inhibitors for the PDE4A5 may have important therapeutic and pharmacologic properties (McPhee et al., 2001; Cherry and Davis, 1999; Perez-Torres et al., 2000).
4.4. Conclusions

In this study rolipram or Prozac administration showed a trend in reducing without statistical significance the obesity-induced depression phenotype. Interestingly, rolipram injections resulted in resistance to dietary-induced obesity phenotype. On the contrary, Prozac daily injections increased body weight and induced liver enlargement compared to saline-injected mice. HFD increased PDE4 activity at the membrane fraction specifically in the hypothalamus. This increase was abolished in PDE4Aβ/β mice. However, the ob/ob:PDE4Aβ/β mice showed increased PDE4 activity at the cytosol fraction of hypothalamus and at the membrane fraction of amygdala compared to the ob/ob mice, suggesting a different pathway might be involved in the obesity induced depression phenotype. HFD specifically upregulated the levels of RNA, protein and phosphorylation state of PDE4A5 in the hypothalamus and downregulated the phosphorylation of CREB, a protein regulated by the cAMP signalling pathway. Loss of PDE4A in vivo was able to rescue the dietary or genetic obesity-induced depression phenotype and this effect was independent of the increase in the body weight and anxiety behaviour. Short term exposure to HFD or genetic obesity did not induce any upregulation of inflammatory cytokines, such as TNF-α, IL-1β or IFN-γ. No differences could be measured in the RNA levels of BDNF (a major neurotrophin that has been linked with depression), when comparing WT and PDE4Aβ/β mice either on ND or HFD, or ob/ob and ob/ob:PDE4Aβ/β. Phosphorylation of DARPP-32 another protein that has been involved in the cAMP/PKA signalling pathway was not affected after consumption of HFD, suggesting other molecular targets being affected downstream the cAMP signalling cascade. The results in this study identify for the first time PDE4A5 as a major molecular player in obesity-induced depression phenotype. The exact molecular mechanisms need to be elucidated. These data suggest that specific targeting of PDE4A5 in the hypothalamus might represent a novel therapeutic target for the treatment of depression in overweight and obese individuals. Isoform-specific PDE4A5 inhibitors will be challenging to develop due to the identical sequence of PDE4 catalytic units. A novel strategy to target the cellular location rather than catalytic activity of PDE4A5 may prove beneficial as a specific pool of PDE4A5 located in the hypothalamic membrane seems to be important in this regard (Lee et al., 2013).
Chapter 5

Specific dietary fatty acids induce a depression phenotype via the activation of free fatty acid receptors in the hypothalamus
5.

5.1. Introduction

The communication between the periphery and the brain is a complex and selective process that is strictly regulated by the blood brain barrier (BBB). The BBB allows only certain molecules to access the brain either for energy supply or the regulation of various signalling pathway processes while preventing other molecules from entering, in order to protect the brain from the damaging factors.

Free fatty acids (FFAs) are not only essential nutritional components, but they can also function as signalling molecules (Cao et al., 2008; Itoh et al., 2003; Nunez, 1997a; Haber et al., 2003; Coppack et al., 1994). They can cross the BBB (Smith and Nagura, 2001) to regulate different molecular pathways in the brain (Cao et al., 2008; Itoh et al., 2003; Nunez, 1997a; Haber et al., 2003; Coppack et al., 1994). The mechanisms that underpin signal transduction from free fatty acids involve the activation of canonical GPCR receptor and PPAR-γ, signal transmission and targeted cell response (Lefkowitz, 2004).

Depression rates vary between different countries (Kessler and Bromet, 2013) and cultures, and this variation might reflect the variation of food habits, especially the intake of dietary fats. Even though there is a correlation between the consumption of different fatty acids and prevalence of depression (Sanchez-Villegas et al., 2011), the molecular mechanisms behind this phenomenon are unknown.
5.1.1. Western diet consumption positively correlates with depression phenotype in humans and rodents, while Mediterranean diet has a protective antidepressant effect

The rising prevalence of depression has been paralleled by a dramatic change in the sources of fat in the diet of the westernized world (Pawels and Volterrani, 2008). Western diet, commonly consumed in northern Europe and USA, is characterized by high caloric consumption, processed food and by the replacement of polyunsaturated (PUFA) or monounsaturated fatty acids (MUFA) by saturated fatty acids (SFA) and trans-unsaturated fats (TFA) (Pawels and Volterrani, 2008). A positive correlation between the development of depression and the consumption of trans fat, as well as an inverse relationship for PUFA and MUFA intake and depression has been described (Sanchez-Villegas et al., 2011).

Epidemiological studies suggest that consumption of the western diet can be a risk factor for depression later in life (Jacka et al., 2010). In accordance with human studies, using a rat strain prone to depression revealed that consumption of a high fat diet, but not a high caloric diet, exacerbated the depressive phenotype as shown in depression related paradigms (Abildgaard et al., 2011). This suggests that the specific diet, rather than the caloric intake, was responsible for the depression phenotype.

While a highly processed food diet can be a risk factor for depression, the consumption of a whole food diet appears to have a protective effect (Akbaraly et al., 2009). Countries located in the southern Europe differ from the northern mainly in the consumption of two major food items: olive oil and pulses (Naska et al., 2006). Higher adherence to the Mediterranean diet, which is rich in legumes, fruits, vegetables, fish and cereals but low in meat and dairy products, was found to be inversely associated with the risk of depression (Sanchez-Villegas et al., 2009).
5.1.2. Fatty acids as signalling molecules in the periphery and the brain

It is now becoming clear that ‘all fats are not created equal’; different fatty acids have distinct roles in the cells and can differentially alter the signalling pathways in various ways. Fatty acids can either bind to their free fatty acid receptors or activate intracellular proteins such as the peroxisome proliferator-activated receptor proteins (PPARγ) (Krey et al., 1997). Figure 5.1 represents the different organs that dietary fatty acids can alter after their entrance in our body.

Figure 5.1. Schematic of the different pathways affected by dietary fatty acids. Figure adapted from: Lam, Schwartz and Rossetti Hypothalamic sensing of fatty acids Nature Neuroscience, 2005

FFAs take part in many peripheral physiological processes, including processes in fat (Li et al., 2010b) and in the pancreas (Itoh et al., 2003; Briscoe et al., 2003; Kotarsky et al., 2003; Salehi et al., 2005; Nolan et al., 2006). Ursolic acid has been shown to stimulate lipolysis in primary cultured rat adipocytes
via the PKA/HSL pathway (Li et al., 2010b). Saturated fatty acids, such as stearic and palmitic acid, stimulate MCP-1 secretion, while palmitoleic leads to resistin secretion in differentiated adipocytes (Schaeffler et al., 2009). Saturated long chain free fatty acids, such as palmitic and lauric acid, have both been shown to trigger inflammatory signalling in cultured macrophages (Laine et al., 2007; Aronis et al., 2008), whereas short chain fatty acids can alter gastrointestinal mobility by regulating enteric neurons (Soret et al., 2010).

Although many studies have recently investigated the involvement of FFA in the regulation of energy balance, the roles of FFAs on brain signalling remain largely unknown. Circulating fatty acids gain rapid access to the brain, where they equilibrate with neuronal long chain co-A (Miller et al., 1987; Rapoport, 1996). Once in the brain they can be further metabolized in mitochondria, via β-oxidation, incorporated into membranes as phospholipids (Miller et al., 1987; Rapoport, 1996), used as energy nutrients, or act as potent signalling molecules in various cellular processes (Cao et al., 2008; Itoh et al., 2003; Nunez, 1997a; Haber et al., 2003; Coppack et al., 1994). The role of FFAs in the brain has mostly been studied in the hypothalamus, as it is the brain region responsible for the regulation of energy homeostasis, and it has been shown that infusion of different fatty acids in this area produces many effects in the periphery including hepatic glucose production (Ross et al., 2010). Centrally administrated fatty acids can affect signalling related to energy balance and body metabolism (Schwinkendorf et al., 2011; Obici et al., 2002; Benoit et al., 2009; Milanski et al., 2009; Velloso et al., 2008). Palmitic acid administration causes brain insulin resistance (Posey et al., 2009), whereas oleic acid acts as a central insulin mimetic, and has a beneficial role on hepatic glucose homeostasis and body weight regulation (Obici et al., 2002). Palmitoleic acid, a lipid that belongs to the group of lipokines, can act as a hormone, as it has been shown to influence energy metabolism (Cao et al., 2008). Palmitoleic acid play a pivotal role in orchestrating the communication of peripheral organs to regulate systemic metabolic homeostasis with hypothalamus and its dysfunction leads to obesity, insulin resistance, diabetes and hyperlipidemia (Cao et al., 2008).
The direct effect of administration of different fatty acids in the hypothalamus has been extensively studied to assess their role in the regulation of appetite. Intracerebrovascular (i.c.v.) injections of either oleic or DHA reduces food intake and body weight by increasing levels of POMC and NPY in the hypothalamus (Schwinkendorf et al., 2011; Obici et al., 2002). In accordance with this finding, central i.c.v. administration of oleic acid has been shown to increase POMC neuronal excitability (Jo et al., 2009). On the other hand, i.c.v. infusion of the saturated fatty acid palmitic acid has been shown to have an orexigenic effect and promote weight gain, increased local inflammation, and impair hypothalamic leptin and insulin signalling (Benoit et al., 2009; Milanski et al., 2009; Velloso et al., 2008). Acetate, following intraperitoneal injection (i.p.), is able to cross the BBB and enter the brain where it can induce changes in the expression profiles of regulatory neuropeptides that favour appetite suppression in the hypothalamus (Frost et al., 2014). Furthermore, in addition to their roles in the hypothalamus, unsaturated fatty acids, such as oleic, linoleic and linolenic (Ikeuchi et al., 1996; Nishizaki et al., 1998), as well as saturated fatty acids, such as stearic acid (Ohta et al., 2003), can facilitate hippocampal synaptic transmission via the PKC signalling pathway. However, even though it has been shown that lipids can have various effects in the brain, the specific molecular pathways, as well as the receptors responsible for their actions are unknown.

Fatty acids can have a direct or indirect effect on neurons. Medium from palmitic acid treated astrocytes may lead to an upregulation of BACE1, a protease important in formation of myelin sheaths, and accumulation of amyloid precursor proteins (APP) in cortical neurons (Patil et al., 2006). In support of the indirect effect, hypothalamic cell lines exposed to saturated fatty acids do not reveal any inflammatory activation or insulin resistance even though they exhibit endoplasmic reticulum stress and apoptosis (Choi et al., 2010). However, even though fatty acids can act as signalling molecules, very little is known about the molecular cascades that they activate to have this effect.
5.1.3. The role of FFAR1 (GPR40) in the brain signalling pathways

To date, Free Fatty Acid Receptor 1 (FFAR1), also known as GPR40, is mostly associated with insulin secretion from pancreatic β-cells (Itoh et al., 2003; Briscoe et al., 2003; Kotarsky et al., 2003; Salehi et al., 2005; Nolan et al., 2006). Very few studies have investigated its role in the brain despite its high expression levels (Briscoe et al., 2003; Ma et al., 2007). GPR40 expression in the hypothalamus has been shown to be involved in the pain regulatory system (Nakamoto et al., 2012). GPR40 was found in the hippocampal neurogenic niche and GPR40 levels increased in this area after ischemia, implying a role for GPR40 in neurogenesis (Ma et al., 2008b). This effect is speculated to be mediated via DHA binding on GPR40, as DHA is a representative high-affinity GPR40 ligand that promotes hippocampal neurogenesis (Kawakita et al., 2006; Yamashima, 2008). This effect is believed to be facilitated by increased phosphorylation of CREB after ischemia, and it was correlated with enhanced hippocampal neurogenesis (Boneva and Yamashima, 2012). Furthermore, arachidonic acid and DHA was recently found to increase intracellular Ca^{2+} levels through activation of GPR40/PLC/IP3 signalling pathway in PC12 cells (Yamashima, 2008) or rat fetal brain neural stem cells that were transfected with the GPR40 gene (Ma et al., 2010). These findings implicate GPR40 in adult neurogenesis of primates and suggest a key role of GPR40 for the action of PUFAs.

5.1.4. HFD consumption induces hypothalamic inflammation

In addition to hormonal signals coming from the periphery, such as leptin and insulin, nutrients such as lipids can cross the BBB and affect the central control of food intake and energy expenditure. This hypothalamic nutrient sensing plays a critical role in the complex network of signals controlling energy balance (Le Foll et al., 2009; Moran, 2010; Cota et al., 2007). Increased dietary fat engages inflammatory processes in the hypothalamus.
that undermine the efficacy of central nutrient sensing mechanisms critical for negative feedback control (De Souza et al., 2005). Hypothalamic sensing of glucose has been proposed to be an important intervention for the treatment of diabetes (Lam et al., 2005a). It has been observed that as early as little as 1 to 3 days on HFD is sufficient to induce hypothalamic inflammation along with reactive gliosis which involves recruitment of microglia and astrocytes (Thaler et al., 2012).

It has been suggested that central availability of dietary fat per se rather than the absolute energetic content of consumed food may promote obesity, central insulin insensitivity and hypothalamic inflammation (Posey et al., 2009). Diets high in fat content and refined carbohydrates are known as risk factors for metabolic syndrome and they induce insulin resistance in rodents (Storlien et al., 1986; Pagliassotti and Prach, 1995; Oakes et al., 1997). Dietary or genetic induced obesity leads to hypothalamic inflammation (De Souza et al., 2005; Milanski et al., 2009; Moraes et al., 2009) and addition of unsaturated fatty acids in the diet can reverse the hypothalamic dysfunction (Cintra et al., 2012).

In summary, a variety of studies has shown a positive correlation between the consumption of the western diet and the development of mood disorders such as depression. On the other hand, the beneficial and protective role of diets rich in PUFAs has been documented in numerous studies. The molecular mechanisms that involve the regulation of mood disorders by the consumption of different diets with different fatty acid composition are unknown. However whether specific dietary fatty acids in the western diet can play a causative role in the development of depression has never been investigated.
5.1.5. Aims

Given the epidemiological and clinical data linking western data and depression it was hypothesized that specific dietary fatty acids may induce a depression phenotype and this effect might happen via the cAMP/PDE4A/PKA signalling pathway in the CNS. Hence, this chapter will focus on the role of different dietary fatty acids in the development of depression and the molecular pathways that are affected.

The aims of this chapter are as follows:

1) to assess the role of specific fatty acids in the development of depression and on PKA signalling
2) to identify in the hypothalamic area the molecular mechanism(s) of the dietary obesity induced depression phenotype

5.2. Results

5.2.1. HFD specifically alters the hypothalamic fatty acid composition

To investigate whether diet can alter the fatty acid composition in the brain, fatty acid profile analysis was performed in the hypothalamic and cortical brain regions of mice fed ND and HFD (short-term and longer-term). Hypothalamic samples were compared to cortical samples, as there was no difference found in the cortex linking diet with depression in this study. Hypothalamic and cortical samples were collected from the same mice and analysed by mass spectrometry. Interestingly, there was an accumulation of most of the fatty acids that were analysed in the hypothalamic area after the consumption of either 4 or 8 weeks (short-term and longer-term, respectively) on HFD compare to mice fed ND (Fig. 5.2). This effect was specific for the hypothalamus as there were no differences in the fatty acid composition in the cortex for mice fed either ND or HFD (Fig. 5.2). This suggests that fatty acid composition in the hypothalamus is specifically affected by HFD consumption compared to other brain region analysed in the same mice.
Figure 5.2. Fatty acid profile analysis for hypothalamic and cortical samples of mice fed either ND or HFD. Heatmaps for the different fatty acids of hypothalamic and cortical samples of mice fed ND versus either 4 or 8 weeks on a HFD. The heatmaps were constructed converting the actual values (µgr) of the different fatty acids into log₂ values. The heatmaps of the fatty acids were divided in two groups due to the high or low representation of the different fatty acids in the brain (n=3-4, analysed by 2-way ANOVA with Bonferroni post-hoc test). The mass spectrometry experiment was carried out by Dr Michelle Puchowicz.

5.2.2. Different fatty acids can differentially affect the forskolin induced activation of PKA signalling

As described in Chapter 1 fatty acids can be divided into two major groups: saturated and unsaturated fatty acids (Schematic 1.4). The opposing roles of the two groups of fatty acids has been shown in many signalling pathways, with unsaturated fatty acids having a beneficial role and saturated fatty acids having a detrimental role (Almaguel et al., 2009; Posey et al., 2009; Obici et al., 2002). Central i.c.v. administration of fatty acids have distinct effects on energy metabolism, neuropeptide expression and food intake regulation, with
unsaturated fatty acids eliciting a more anorexigenic response compared with the saturated fatty acids (Schwinkendorf et al., 2011; Obici et al., 2002; Benoit et al., 2009; Milanski et al., 2009; Velloso et al., 2008). However, the molecular mechanism of these effects is unknown. To investigate the role of different fatty acids on PKA signalling, fluorescence resonance energy transfer (FRET) experiments were performed in a mouse neuroblastoma cell line (N2a) treated with different fatty acids. N2a cells were transfected with a FRET PKA sensor that detects the activation of PKA signalling. Figure 5.3. is a schematic representation of the FRET PKA sensor used in this study. This sensor consists of the regulatory subunit I of the PKA protein and is cloned between the YFP and the CFP protein. N2a cells were co-transfected with PDE4A5 as it was shown to play an important role in obesity induced depression phenotype along with β-arrestin 2. Arrestins act as scaffolding proteins to facilitate the interaction between a GPCRs and intracellular molecules (Shenoy and Lefkowitz, 2005). At the end of all FRET experiments, cells were treated with high concentration of forskolin (stimulation of cAMP production) and with 3-isobutyl-1-methylxanthine, IBMX (inhibition of cAMP degradation) in order to detect FRET signal and verify that cells are still responsive to different treatments.

Figure 5.3. Schematic of the PKA FRET sensor. Represents a schematic of the FRET PKA sensor used in this study. This sensor consists of the regulatory subunit I of the PKA protein and is cloned between the YFP and the CFP protein. In the absence of cAMP the PKA FRET sensor has a specific structure that allows the CFP and YFP to be in close proximity resulting in high FRET signal. In the presence of cAMP, cAMP can bind to the PKA regulatory subunit
leading to a structural conformation where CFP and YFP are far from each other, leading to low FRET signal. 

**Figure 5.4** shows the structure of the different fatty acids used for the treatment of the neuroblastoma cell line (N2a).

![Chemical formulas of fatty acids](image)

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**Figure 5.4. Chemical formulas of the fatty acids.** Formulas of palmitic, myristic and oleic acid used to treat N2a cells. Palmitic and myristic acid belong to the saturated group while oleic acid belongs to the unsaturated fatty acid group.
Treatment of neuronal cells transfected with the PKA sensor with forskolin led to an increase FRET signal due to the activation of PKA (Fig. 5.5). Forskolin is a diterpine that directly activates adenylyl cyclase to produce cAMP from ATP (Seamon et al., 1981) that in turn binds to the PKA regulatory subunit and induces a structural transformation that leads to PKA activation and FRET signal. Surprisingly, pretreatment of N2a cells with palmitic acid was able to block the forskolin-induced PKA activation observed in the N2a cell line (Fig. 5.5). On the contrary, pretreatment with either oleic acid or myristic acid did not affect the forskolin-induced activation of PKA (Fig. 5.5). The quantification of the above FRET experiment in N2a cells treated with either palmitic, oleic or myristic acid is shown in Fig. 5.6.

Figure 5.5. Palmitic acid specifically abolishes the forskolin induced activation of PKA signaling in neuronal cells. FRET analysis of N2a cell line (mouse neuroblastoma cell line). Forskolin induced activation of PKA signaling is shown alone (A) or following pretreated with 100µM of either (B) palmitic, (C) oleic or (D) myristic acid. Cells were transfected with PDE4A5 and β-arrestin -2 (ARB2). Palmitic but not oleic or myristic acid was able to block the forskolin induced activation of PKA. (n=6-8 per condition). This experiment was carried out by Dr Nicola Devine.
Figure 5.6. Palmitic acid specifically abolishes the forskolin induced activation of PKA signalling in neuronal cells. Quantification of the FRET experiment done in N2a cell line (mouse neuroblastoma cell line) pretreated with either 100uM of either palmitic, oleic or myristic acid. Palmitic but not oleic or myristic acid was able to block the forskolin induced activation of PKA. (n=6-8, ***P < 0.001 by 1 way ANOVA with Dunnett’s multiple comparison test). This experiment was carried out by Dr Nicola Devine.

5.2.3. Increased consumption of palmitic acid may induce a depression phenotype in mice

Many studies support the beneficial and protective role of unsaturated fatty acids in depression (Mozaffarian et al., 2010; Fernandez-Jarne et al., 2002; Kontogianni et al., 2007; Barzi et al., 2003). Little is known, however, about the detrimental effect of specific dietary fatty acids in depression, and whether they can play a causative role in the development of depression. To investigate whether high consumption of palmitic acid induces a depression phenotype in mice, we decided to administer, via oral gavage, high levels of palmitic acid. Ghee, (clarified butter) consists of a very high percentage of palmitic acid (Mirghani et al., 2010). As a control we administered olive oil,
which consists of high levels of oleic acid (Mirghani et al., 2010). Table 5.1 shows the different fatty acid composition of both ghee and olive oil. Ghee consists of almost 40% of palmitic acid. In contrast, olive oil consists of just 15% palmitic acid.

Table 5.1. Fatty acid composition of ghee and olive oil. Different percentages of the different fatty acids in ghee and olive oil.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>%</th>
<th>Ghee</th>
<th>%</th>
<th>Olive oil</th>
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<tr>
<td>Saturated Fatty Acid</td>
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<tr>
<td>C4:0 Butyric Acid</td>
<td>0.70%</td>
<td></td>
<td>65%</td>
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<tr>
<td>C6:0 Caproic Acid</td>
<td>2.20%</td>
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<td></td>
<td></td>
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<tr>
<td>C8:0 Caprylic Acid</td>
<td>1.20%</td>
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<td></td>
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<tr>
<td>C10:0 Capric Acid</td>
<td>2.50%</td>
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<tr>
<td>C12:0 Lauric Acid</td>
<td>2.70%</td>
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<tr>
<td>C14:0 Myristic Acid</td>
<td>11.80%</td>
<td></td>
<td>0.1-1.2%</td>
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<tr>
<td>C16:0 Palmitic Acid</td>
<td>32-36.70%</td>
<td></td>
<td>7-16%</td>
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<tr>
<td>C18:0 Stearic Acid</td>
<td>12.50%</td>
<td></td>
<td>1-3%</td>
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<tr>
<td>Monounsaturated Fatty Acid</td>
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<tr>
<td>C16:1 Palmitoleic Acid</td>
<td>3.30%</td>
<td>32%</td>
<td>65-80%</td>
<td>77%</td>
</tr>
<tr>
<td>C18:1 Oleic Acid</td>
<td>26.20%</td>
<td></td>
<td></td>
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<tr>
<td>C20:1 Gadoleic Acid</td>
<td>1.20%</td>
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<tr>
<td>C22:1 Eruciic Acid</td>
<td>0.20%</td>
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<tr>
<td>Polyunsaturated Fatty Acid</td>
<td>3%</td>
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<td>9%</td>
<td></td>
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<td>Total %</td>
<td>100%</td>
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Thus the consumption of high levels of palmitic acid was tested *in vivo* in order to see whether it can induce a depression phenotype. To test this hypothesis, mice were orally gavaged twice per day for either three or seven weeks with either ghee or olive oil. **Fig. 5.7** shows the experimental design for the oral gavage administration of either ghee or olive oil for either 3 or 7 weeks. Both ghee and olive oil were pre-warmed at 37°C for 30 minutes before the administration.

**Figure 5.7. Experimental design for the oral gavage with oils.** Schematic of the experimental design for the mice that were gavaged with either ghee or olive oil. The mice were gavaged with 300ul (0.3g FA≈3kcal) twice per day for 3 or 7 weeks.
Following three weeks of oral gavage, there was no difference in the depression phenotype, as assessed with either TST or SPT between mice that received ghee (high palmitic acid) and mice that received olive oil (low palmitic acid) as shown (Fig. 5.8) and (Fig. 5.9).

Figure 5.8. Ghee consumption does not alter the immobilization time at the tail suspension test after 3 weeks on gavage. Immobilization time at the tail suspension test for mice that were gavaged for 3 weeks with either olive oil or ghee (n=9).

Figure 5.9. Ghee consumption does not affect the sucrose consumption at the sucrose preference test after 3 weeks. Sucrose preference test for mice that were gavaged for 3 weeks with either olive oil or ghee (n=8-9). One mouse per group was excluded as they both had a measurement of zero consumption in one of the two bottles.

Interestingly, a pilot experiment after seven weeks of gavage showed a trend towards increasing the immobilization in the tail suspension test in ghee gavaged mice compared to olive oil gavaged mice (Fig. 5.10). Following this
longer period of gavage, mice gavaged with ghee tended to consume less sucrose compare to the olive oil gavaged mice (Fig. 5.11). In Fig. 5.11 as shown by the plot there was one mouse from each group that consumed much less sucrose than the rest in each group. These mice were outliers according to the z score test, Thompson Tau, Grubb’s test and mild outlier criteria of the IQR test. Further excluding these values reveals a significant (P<0.01) decrease of sucrose consumption for the ghee gavaged mice compare to olive oil. However, further studies with bigger cohorts should be done in order to establish whether ghee induces a depression phenotype in mice compared to olive oil. This effect was not related to body weight, as both groups showed the same body weight, with a trend that ghee gavaged mice gaining weight after the seventh week on the gavage (Fig. 5.12).

Figure 5.10. Ghee consumption tended to increase the immobilization time at the tail suspension test. Immobilization time during the tail suspension test for mice that were gavaged for 7 weeks with either olive oil or ghee (n=9).

Figure 5.11. Ghee consumption tended to decrease the sucrose consumption at the sucrose preference test. Sucrose consumption for mice that were gavaged for 7 weeks with either olive oil or ghee (n=9-10).
Figure 5.12. Daily oral gavages of either olive oil or ghee does not affect the body weight. Body weight curves of WT mice that were gavaged twice per day for 8 weeks with either olive oil or ghee on ND (n=9-10).

5.2.4. Single stereotactic injection of palmitic acid in the hypothalamus induces a depression phenotype in vivo

Next, it was hypothesized that palmitic acid can directly signal in the CNS to induce a depression phenotype. Therefore, the direct effect of palmitic acid in the hypothalamic area was tested. In a pilot study with just 3 mice per group bilateral single stereotactic injections of 10µg of palmitic acid in the hypothalamic area revealed an increase in the immobilization time of the tail suspension test compared to the mice that received either artificial cerebrospinal fluid (aCSF) or ethanol injections as control (Fig. 5.13). The single bilateral stereotactic injections did not affect the rearing or the total levels of activity of the mice at the open field test (Fig. 5.14) suggesting that the depression phenotype develops independently of the total levels of activity. This pilot experiment needs to be repeated and better solvent of palmitic acid should be used as ethanol has been shown to affect depressive behaviours in mammals.
Figure 5.13. Single injection of palmitic acid induces an increase at the immobilization time at the tail suspension test. Immobilization time at the tail suspension test of mice that received either 2ul of aCSF, 2ul of 2% ethanol or 2µl of 5µg/µl of palmitic acid dissolved in ethanol. (n=3 1-WAY ANOVA **P<0.01,*P<0.05) Stereotactic injections and immobilization quantification was performed by Dr Jae Kuy Ruy.

Figure 5.14. Single injection of palmitic acid does not alter the total activity at the open field test. Open field test for WT mice that received a single injection of either aCSF, ethanol of palmitic acid (n=3).

5.2.5. Dietary or genetic obesity induces an upregulation of fatty acid receptors

As it has been mentioned before, fatty acids exert their effect by binding to appropriate fatty acid receptors to regulate intracellular processes. Therefore, the role of different free fatty acid receptors in the hypothalamus
for this model of depression was investigated. To investigate the involvement of different fatty acid receptors in the hypothalamic area, their RNA levels were measured with RT-PCR. Real time PCR data revealed an increase of the GPR41 RNA levels after 3 weeks on HFD (Fig. 5.15).

**Figure 5.15.** Dietary obesity tends to increase the RNA levels of GPR41 in the hypothalamus. Real-time PCR analysis of GPR41 in the hypothalamus of WT mice on ND, 1 week and 3 weeks on HFD and ob/ob mice (n=4).

The RNA levels of the GPR120 receptor were up-regulated in the hypothalamic area of the genetically obese (ob/ob) mice (Fig. 5.16).

**Figure 5.16.** Genetic obesity increases the RNA levels of GPR120 in the hypothalamus. Real-time PCR analysis of GPR120 in the hypothalamus of WT mice on ND, 1 week and 3 weeks on HFD and ob/ob mice (n=4, *P < 0.05 , by one-way ANOVA with Tukey’s multiple comparison test).
Most strikingly though, the levels of GPR40 RNA were upregulated 4-5 fold in WT mice after just one week on HFD and for the genetically obese mice (Fig. 5.17). These data suggest that the diet can change the levels of different free fatty acid receptors in the brain of WT and ob/ob mice. However at the protein level, HFD did not induce any change in the level of GPR40 (Fig. 5.18).

Figure 5.17. Dietary or genetic obesity increases the RNA levels of GPR40 in the hypothalamus. Real-time PCR analysis of GPR40 in the hypothalamus of WT mice on ND, 1 week and 3 weeks on HFD and ob/ob mice (n=4, *P < 0.05 , by one-way ANOVA with Bonferroni multiple comparison test).

Figure 5.18. High fat diet does not affect the total protein levels of GPR40 in the hypothalamus. Western blot analysis for total levels of GPR40 in the hypothalamus. GAPDH was used as a loading control (n=4).
5.2.6. No differences at the RNA levels of arrestins in the hypothalamus

As mentioned above, free fatty acids receptors are all GPCRs (Brown et al., 2005). β-arrestins can interact with GPCRs in order to regulate various GPCRs and intracellular signal pathways (Gurevich et al., 1995) (Palczewski, 1994). Real time PCR analysis, however, revealed no differences for transcript levels of either β-arrestin 1 (Fig. 5.19) or β-arrestin2 (Fig. 5.20). Moreover, no differences in the total levels of β-arrestin2 were detected in the hypothalamus of mice fed either ND or HFD (Fig. 5.21).

![Figure 5.19](image)

**Figure 5.19.** No differences in the RNA levels of β-arrestin1 after dietary or genetically induced obesity. Real-time PCR analysis of β-arrestin 1 for WT, PDE4A^{-/-}, ob/ob and ob/ob:PDE4A^{-/-} on either ND, 1 and 3 weeks on HFD (n=4)
Figure 5.20. No differences in the RNA levels of beta-arrrestin 2 after dietary or genetically induced obesity. Real-time PCR analysis of beta-arrrestin1 for WT, PDE4A$^{+/+}$, ob/ob and ob/ob: PDE4A$^{+/+}$ on either ND, 1 and 3 weeks on HFD (n=4).

Figure 5.21. High fat diet does not affect the total protein levels of β-arrrestin2 in the hypothalamus. Western blot analysis for total levels of β-arrrestin 2 in the hypothalamus. GAPDH was used as a loading control (n=4).
5.2.7. GPR40 interacts with PDE4A5 in the hypothalamus

Previously it was shown that dietary or genetic obesity induces the upregulation of PDE4A5 in the hypothalamus (Fig. 4.13 & 4.14). Therefore, I tested whether GPR40 can interact with PDE4A5 in vivo. Studies have shown that members from the PDE4 family can interact with GPCRs (Perry et al., 2002). To investigate the possible interaction of PDE4A5 and GPR40, co-immunoprecipitation analysis was performed using extracts from the hypothalamus of mice fed either ND or HFD. Immunoprecipitation against GPR40 and western blotting for PDE4A5 revealed a putative interaction of PDE4A5 with GPR40 in the hypothalamus of mice fed either ND or HFD (Fig. 5.22).

Figure 5.22. GPR40 interacts with PDE4A5 in the hypothalamus of mice fed either normal or high fat diet. Immunoprecipitation of 700ug of hypothalamic samples from mice (fasted for 7h, and then food was added for 2h) fed either ND or HFD with the GPR40 rabbit antibody (Santacruz) and western blotting with PDE4A5. (n=1)

5.2.8. GPR40 interacts with PDE4A5 in vitro

Next we wanted to see whether we could recapitulate the effects of the dietary fatty acids and confirm this interaction in vitro. Fats can be purchased with a methyl, ethyl or acid group but the fatty acid group was used as it has been shown that the carboxyl group is indispensable for this function as the methyl group of fats does not induce any calcium changes after GPR40 activation (Itoh et al., 2003). For the in vitro treatments, the mouse neuroblastoma cell line (N2a) was employed as it has been shown to express
high levels of GPR40. Previous studies have shown that members from the PDE4 family can interact with GPCRs in an arrestin dependent or independent way (Richter et al., 2008). Co-Immunoprecipitation analysis of transiently transfected N2a cells with PDE4A5 and β-arrestin 2 (ARB2) revealed a possible interaction between PDE4A5 and GPR40 in basal levels and after 5 minutes of palmitic acid treatment (Fig. 5.23).

![N2a in vitro Co-IP](image)

**Figure 5.23.** PDE4A5 interacts with GPR40 in neurons *in vitro.* Co-immunoprecipitation between GPR40 and PDE4A5 in N2a cell line after 5 minutes with 500µM of palmitic acid stimulation. Immunoprecipitation was performed with 1.3mg of protein and using the PDE4A antibody from abcam. Cells well transiently transfected with PDE4A5 and ARB1 and ARB2. (n=1)
Next I examined whether the GPR40-PDE4A5 interaction happens in HEK293 cells. HEK293 cells transiently transfected with PDE4A5-vsv, GPR40-flag and β-arrestin 2 revealed a possible interaction between PDE4A5 and GPR40 (Fig. 5.24). HEK293 cells were treated at different time points with 500µM of palmitic acid and immunoprecipitation analysis was performed. Interestingly, the interaction was time dependent to the palmitic acid treatment (Fig. 5.24).

![Figure 5.24. PDE4A5 interacts with GPR40 in vivo in HEK293 cells. Co-immunoprecipitation of PDE4A5 with GPR40 in HEK293 cells treated in different time points with 500µM of palmitic acid. Immunoprecipitation was performed with 50µgr per condition. HEK293 cells were transiently transfected with PDE4A5-vsv, ARB2 and GPR40-flag.](image)

Palmitic acid is a fatty acid that exists in abundance in the fetal bovine serum in which cells grow. Therefore, we decided to investigate whether this interaction would be enhanced after serum deprivation of HEK293 cells. The same experiment was performed but this time the cells were deprived for either 5h (Fig. 5.25) or 12h (Fig. 5.26) prior to the treatment with palmitic acid. Co-immunoprecipitation analysis of HEK293 cells revealed a possible interaction between PDE4A5 and GPR40 at basal as well as at different time points after treatment with palmitic acid (Fig. 5.25) and (Fig. 5.26). The immunoprecipitation with the FLAG antibody was not the same for all the different conditions and therefore there was an unequal interaction with PDE4A5 for (Fig. 5.25) and (Fig. 5.26). Moreover, either 5 or 12 hours of...
serum deprivation did not affect the interaction between PDE4A5 and GPR40 (Fig. 5.25) and (Fig. 5.26).

**Figure 5.25.** PDE4A5 interacts with GPR40 *in vitro* in HEK293 cells. Co-immunoprecipitation of PDE4A5 with GPR40 in HEK293 cells treated in different time points with 500uM of palmitic acid. Immunoprecipitation was performed with 750ugr per condition. HEK293 cells were transiently transfected with PDE4A5-vsv, ARB2 and GPR40-flag. The HEK293 cells were deprived from serum for 5h prior to the treatments with palmitic acid. (n=1)

**Figure 5.26.** PDE4A5 interacts with GPR40 *in vitro* in HEK293 cells. Co-immunoprecipitation of PDE4A5 with GPR40 in HEK293 cells treated in different time points with 500uM of palmitic acid. Immunoprecipitation was performed with 750ugr per condition. HEK293 cells were transiently transfected with PDE4A5-vsv, ARB2 and GPR40-flag. The HEK293 cells were deprived from serum for 12h prior to the treatments with palmitic acid. (n=1)
5.2.9. Palmitic acid treatment of neuronal cells increases the PDE4A5 translocation at the membrane fraction

Previously I showed that the compartmentalization of phosphodiesterase activity is important for the dietary obesity induced depression phenotype in the hypothalamus (Fig. 4.19). To investigate whether palmitic acid treatment changes the compartmentalization of PDE4A5 in this *in vitro* system, N2a cells were treated with different concentrations of palmitic acid for 5 minutes. Interestingly, there was a concentration dependent increase of the PDE4A5 protein accumulation at the membrane fraction of N2a cells treated with different concentrations of palmitic acid (Fig. 5.27).

![Concentration dependent increase of PDE4A5 at the membrane fraction of neuronal cells.](image)

**Figure 5.27.** Concentration dependent increase of PDE4A5 at the membrane fraction of neuronal cells. Membrane fractionation of N2a cells transfected with PDE4A5 and ARB2 and have been treated with increasing concentrations of palmitic acid for 5 minutes. Concentration dependent increase of PDE4A5 at the membrane fraction. WB was run with 10ugr for the membrane fraction and 5ugr for the cytosol fraction. (n=1)
Next, membrane fractionation of N2a cells treated with palmitic acid in different time points revealed an increase of the PDE4A5 and ARB2 at the membrane fraction after 2 minutes of palmitic treatment (Fig. 5.28).

A similar effect of palmitic acid on HEK293 cells was observed after 5 minutes of treatment with increased phosphorylation as well (Fig. 5.29).
5.2.10. Oleic acid treatment of neuronal or HEK293 cells does not alter the levels of PDE4A5 at the membrane fraction

GPR40 has been shown to bind both saturated and unsaturated fatty acids. We thus set out to investigate whether oleic acid, which is an unsaturated fatty acid, has the same affects as palmitic acid. Interestingly, the same concentration of oleic treatment in N2a cells did not affect the localization of the PDE4A5 (Fig. 5.30). In accordance, treatment of HEK293 cells with oleic acid in different time points did not affect the protein levels of PDE4A5 at the membrane fraction (Fig. 5.31).

Figure 5.30. Oleic acid treatment does not alter the levels of PDE4A5 and ARB2 at the membrane fraction in N2a cells. Membrane fractionation of N2a cells treated in different time points with 500uM of oleic acid. (n=1)
5.2.11. GPR40 agonist treatment does not change the protein levels of PDE4A5 at the membrane fraction

Palmitic acid has been shown to be a ligand for GPR40. However it has also been shown to stimulate other cell membrane receptors as well as intracellular receptors. Therefore a specific GPR40 agonist was used to determine whether the specific activation of GPR40 induces the translocation of PDE4A5 at the membrane fraction. GW9508 is a novel GPR40/GPR120 agonist that is 100 fold more selective for GPR40 rather than GPR120 (Briscoe et al., 2006). GW9508 has a higher affinity for GPR40 than the other fatty acids ligands do (Briscoe et al., 2006). Fig. 5.32 shows the structure of the GPR40 agonist used in this study.
Treatment with either low (10 µM) or high (100 µM) concentrations of the GPR40 agonist did not alter the levels of PDE4A5 at the membrane fraction (Fig. 5.33) and (Fig. 5.34).

**Figure 5.33.** GPR40 agonist does not induce any PDE4A5 accumulation at the membrane fraction. Membrane fractionation of HEK293 cells treated with 10µM of GW-9508, a selective GPR40 agonist. HEK293 cells transfected with PDE4A5, ARB2 and GPR40. 10µgr of the membrane fraction and 10µgr of the cytosol fraction were used for the western blot.

**Figure 5.34.** GPR40 agonist does not induce any PDE4A5 accumulation at the membrane fraction. Membrane fractionation of HEK293 cells treated with 100µM of GW-9508, a selective GPR40 agonist. HEK293 cells were transiently transfected with PDE4A5, ARB2 and GPR40. 10µgr of the membrane fraction and 5µgr of the cytosol fraction were used for the western blot.
5.2.12.

5.2.13. *In vivo* administration of the GPR40 antagonist rescues the dietary obesity induced depression phenotype

Next we set out to determine whether the selective blocking of GPR40 *in vivo* would rescue the dietary obesity-induced depression phenotype. GW1100, a selective antagonist for the GPR40 was identified by others previously using high-throughput screening (Zhao et al., 2011). GW1100 is a selective GPR40 antagonist with the exception of oxytocin receptor that acted as an antagonist as well (Briscoe et al., 2006). **Fig. 5.35** shows a schematic of this GPR40 antagonist as well as the experimental design for the continuous pump infusion of GPR40 antagonist in the third ventricle of mice fed a HFD.

**Figure 5.35.** Experimental design for the pump implantations for the *in vivo* administration of GPR40 antagonist. Schematic of the experimental design for the mice that received GPR40 antagonist in the third ventricle via i.c.v. pump implantation. Mice were on HFD for three weeks with the last two weeks being administered the GPR40 antagonist.
Interestingly, a pilot experiment revealed a dose-dependent reduction of the immobilization time of the TST of mice that received two different concentrations of GPR40 antagonist compare to the mice that received aCSF control infusion (Fig. 5.36).

![Bar chart showing immobilization time (s) for different concentrations of GPR40 antagonist](image)

**Figure 5.36.** GPR40 antagonist administration *in vivo* tend to reduce the immobilization time at the tail suspension test. Immobilization time during the tail suspension test for mice that were administered GPR40 antagonist in the third ventricle for 2 weeks while the mice were on a HFD. Mice received two different concentrations of GPR40 antagonist either 0.1µg/µl and 1µg/µl for 2 weeks (n=3). Stereotactic surgeries were performed with Dr Jae Kyu Ryu.
Moreover, a pilot experiment that included a sham control showed that GPR40 antagonist administration tend to reduce the immobilization time during the TST test (Fig. 5.37), suggesting that blocking the activation of the GPR40 might be sufficient to rescue the dietary obesity-induced depression phenotype in these mice.

Figure 5.37 GPR40 antagonist administration in vivo tend to reduce the immobilization time at the tail suspension test. Immobilization time at the tail suspension test for mice that were administered GPR40 antagonist and aCSF in the third ventricle for 2 weeks while the mice were on a HFD for three weeks. Mice were compared with the sham mice that underwent surgery without the implantation of pumps (n=3-11). Stereotactic surgeries were performed with Dr Jae Kyu Ryu.
5.3. Discussion

Developed countries predominantly consume relatively inexpensive high-caloric food that is low in essential nutrients (Pawels and Volterrani, 2008). The increased adoption of a western type diet positively correlates with the development of depression in developed countries as shown in many epidemiological studies (Sanchez-Villegas et al., 2011). The prevalence of depression is markedly different across various countries and this correlates with the dietary differences in these societies (Kessler and Bromet, 2013).

In spite of the high prevalence of mental disorders in modern societies, little etiological research has been conducted to assess the molecular mechanisms that exist in the CNS to link components of nutrition with depression (Payne, 2010). Dietary fatty acids are a major component of the diet. The nutritional differences between fatty acids of different lengths and saturation states make up a great body of conflicting literature. Historically, saturated fatty acids have been seen as detrimental insofar as they induce insulin resistance and other facets of the metabolic syndrome (Riserus, 2008). On the other hand, polyunsaturated fatty acids have been shown to have the opposite effect: several, such as linolenic and conjugated linoleic acid, are protective against the harmful effects of obesity (Barre, 2007; Li et al., 2008; Zhou et al., 2008). However, the molecular mechanisms that link obesity with depression are unknown. The aim of this study was to investigate whether there are specific components of the diet that can be a causative factor for the development of depression. If that’s the case then can we gain an insight into the molecular mechanisms that underlie this phenomenon?
5.3.1. HFD consumption induces a fatty acid influx specifically in the hypothalamus in mice

High fat diet consumption causes changes in the fatty acid composition of brain lipids (Anding and Hwang, 1986). HFD leads to an increase of two long chain acyl-coA species, palmitoyl- and stearoyl-coA in the hypothalamus and this accumulation has been implicated in the development of peripheral insulin resistance (Posey et al., 2009). Essential polyunsaturated fatty acids have been shown to modulate enzymes, the transport and interact with lipid biolayers to affect metabolic pathways (Mies et al., 2007; Ferrier et al., 2002; Kahn-Kirby et al., 2004; Schonfeld et al., 2004). It is further likely that the activity of the different enzymes is altered in pathophysiological conditions and the different fatty acid processes such as desaturation and elongation are different in disease than physiological conditions. When the levels of DHA are low in the brain, its turnover rate is much slower rate than in controls suggesting the existence of a compensatory mechanism (DeMar et al., 2004). Higher levels of saturated fatty acids in the plasma membrane decrease membrane fluidity by reducing the polyunsaturated to saturated fatty acid ratio in the membrane and this alters the binding of the agonist on adrenergic receptors, one of the pathways impaired in depression (Miyamoto et al., 1990; Piletz et al., 1991; Wince and Rutledge, 1981). Mood regulating drugs such as lithium and valproate play a role on the turnover of a long chain fatty acid, arachidonic acid (Chang et al., 1996; Chang et al., 2001). Furthermore, the activity of some brain enzymes has been found to be decreased by mood stabilizing drugs (Chang and Jones, 1998; Ghelardoni et al., 2004; Bosetti et al., 2002; Bosetti et al., 2003). Moreover, the increased arachidonic acid that was observed in the hypothalamus of FSL rats, an animal model of depression, suggests that the differences in the brain fatty acid composition might be a consequence of the disease itself as both FSL and control rats were fed identical diets (Green et al., 2005). Another interesting point is the general lack of knowledge concerning the transport, metabolism and signalling of all these dietary fatty acids or their downstream metabolites after their entrance in the brain. Future studies will be needed to characterize the central
metabolism of these fatty acids and it will be necessary to determine and identify how different dietary patterns can affect the enzymes related to fatty acid oxidation and metabolism.

Interestingly, activation of the cAMP/PKA signalling pathway induces rapid phosphorylation and activation of SIRT1 that in turn deacetylates substrates that can promote lipid oxidation and energy expenditure in vivo (Gerhart-Hines et al., 2011). Moreover, central inhibition of lipid oxidation is sufficient to restore hypothalamic lipid sensing and normalize feeding behaviour and glucose homeostasis (Pocai et al., 2006). In this study I showed a decrease of the cAMP/PKA signalling in response to the HFD (Fig. 3.12&3.13&3.14) suggesting that this decrease of the cAMP/PKA signalling can affect the lipid oxidation in the brain. Moreover, we showed that lipids can affect PKA signalling (Fig. 5.5&5.6). Therefore, detailed examination of the lipids in the brain, as well as the enzymatic pathways of lipid metabolism, in depression can lead to new approaches toward understanding and treating this complex human disorder. Technological advances that allow the assessment of subtle and rapid physiological variation of different fatty acids in the CSF after the consumption of a HFD will no doubt help confirm or refine these observations.

Whether there is a different fatty acid composition in the brain of PDE4A−/− mice that consume HFD compare to mice fed ND should be further investigated. Interestingly it has been shown that phosphodiesterase inhibition can rescue the chronic cognitive deficits produced by traumatic brain injury (Titus et al., 2013). Traumatic brain injury has been linked with increase release of fatty acids (Bazan, 1970; Rehncrona et al., 1982; Zhang and Sun, 1995; Dhillon et al., 1997; Homayoun et al., 1997). Therefore, whether phosphodiesterases control the fatty acid release and composition in the brain is a very interesting topic that deserves further investigation.
The increase influx of fatty acids specifically in the hypothalamus compared to cortex has never been documented before (Fig. 5.3). The hypothalamus is a highly vascularized area with a lot of communication with the periphery. It plays a central role in coordinating physiological responses between the periphery and the central nervous system. The hypothalamus has been studied extensively for its role in appetite regulation as well as regulating energy balance (Cota et al., 2007; Le Foll et al., 2009; Moran, 2010; Lam et al., 2005b; Woods et al., 1998; Schwartz et al., 2000; Ahima et al., 1996; Wang et al., 1998; Obici et al., 2002). However, this is the first study that points out the important role of hypothalamus in linking dietary fatty acids with the development of depression.

The elevation of fatty acids in the hypothalamus, such as palmitic acid, might lead to increase apoptotic events in the hypothalamus (Ulloth et al., 2003). In contrast to this, however, it has been shown that HFD can increase neurogenesis in the hypothalamus (Lee et al., 2012). Further studies investigating the neuronal death or survival of hypothalamic neurons after the consumption of HFD are needed in order to uncover the details of such alterations in neurogenesis. Therefore, determining the consequences of this local accumulation of FFAs after HFD and its potential connection with depression requires further research.

This study also opens a new concept that the BBB is not equally sealed, as some areas of the CNS are more prone to be in contact with the molecules in the vasculature compared to others. Some brain regions, like the hypothalamus, might be more easily accessible compared to others. Future drug development strategies could take advantage of a drug crossing of the BBB in a more easily accessible area such as the hypothalamus, and then, once in the brain, the drug could directed to a different brain region of interest.
5.3.2. Different fatty acids can differentially affect the PKA signalling cascade

Epidemiological and clinical studies support the beneficial and protective role of unsaturated fatty acids in depression whereas western diet consisting highly of trans and saturated fat positively correlates with the development of depression (Sanchez-Villegas et al., 2011), however the molecular pathway of this discrimination is unknown. The data in this study supports a differential role of different fatty acids in the regulation of the PKA signalling (Fig. 5.5&5.6). To our knowledge this is the first time that pretreatment of different fatty acids has been shown to affect forskolin-induced activation of PKA in a neuronal cell line (Fig. 5.5&5.6).

The saturated palmitic acid was able to block the forskolin induced activation of PKA, whereas the unsaturated fatty acid oleic did not affect the PKA activation in vitro in a neuronal cell line (Fig. 5.5&5.6). Interestingly, this blockage of PKA signalling after palmitic treatment is in agreement with the downregulation of PKA signalling after the consumption of HFD (Fig. 3.12&3.13). However, the saturated fatty acid myristic did not affect forskolin-induced activation by PKA in vitro (Fig. 5.5&5.6). This suggests functional differences between saturated fatty acids, as C14-myristic and C16-palmitic did not have the same effect on PKA. While a possible explanation for this effect may be that higher concentration of myristic acid is needed in order to suppress in vitro PKA activation, compared to palmitic, it is also possible that this reflects neuronal specificity of the receptor and the downstream signalling for these different chain length fatty acids. These results suggest that we cannot generalize the two categories and propose that saturated fatty acids are able to down-regulate PKA signalling compared to the unsaturated fatty acids, which do the opposite therefore further understanding on the concentration and specificity of the effect of the different fatty acids is necessary.
It is also possible that fatty acids are acting on the GPR40 protein expressed on the plasma membrane by operating from an intracellular location. Whether palmitic acid binds directly on GPR40 and this result in the intracellular effect on the PKA signalling should be further studied by blocking the receptor first with GPR40 antagonist and then pretreating the cells with palmitic acid.

5.3.3. The beneficial and protective role of olive oil in depression

An inverse association between olive oil consumption and depression has been described (Sanchez-Villegas et al., 2011); However the molecular mechanism of this effect is unknown. Olive oil contains bioactive polyphenols with known anti-inflammatory properties (Waterman and Lockwood, 2007) counting for the anti-inflammatory role of olive oil (Estruch et al., 2006; Salas-Salvado et al., 2008; Mena et al., 2009). Depression has already been described as a disease with high levels of inflammation, suggesting that the trend of the antidepressant effect of olive oil compare to ghee shown at the TST and SPT in this study might be mediated via the anti-inflammatory effect of olive oil (Estruch et al., 2006; Salas-Salvado et al., 2008; Mena et al., 2009). Studies in various cell types (macrophages, adipocytes, myocytes, hepatocytes) have revealed the pro-inflammatory action of saturated fatty acids (Hwang and Rhee, 1999; Nguyen et al., 2005; Yu et al., 2002) and this is mediated at least in part through activation of TLR4/2 (Lee et al., 2001; Nguyen et al., 2007; Schaeffler et al., 2009; Milanski et al., 2009). However, another study suggested that saturated fatty acids do not directly stimulate TLR4 receptors, suggesting an alternative mechanism that might link dietary fat intake with inflammation and they explained the observed effect of previous studies that showed activation due to contamination of fatty acid free BSA with LPS (Erridge and Samani, 2009). Further investigation on the role of inflammation in mice gavaged for 7 weeks as well as the potential suppression of inflammation by olive oil in this model of depression is needed.
One of the components of olive oil is tyrosol, which has been shown to play a role in the intracellular antioxidant defence mechanism (Di Benedetto et al., 2007). One of the pathological characteristics of depression is oxidative stress (Bilici et al., 2001). As it has been shown that the antioxidant defence is decreased among people with depression, maybe olive oil act its antidepressant effect by restoring the antioxidant defence of the body.

Olive oil consists of 80% oleic acid (Waterman and Lockwood, 2007), which is the most abundant fatty acid of myelin, the protective sheath that covers axons. Oleic acid is the precursor of the lipid oleamide that has been shown to play important role in mood disorders (Puri and Richardson, 2000). In addition, it plays an important role in the maintenance of the physiochemical properties of membranes via its ability to increase the δ-9 desaturase enzyme activity (Yehuda et al., 2002) and thus improving the binding of serotonin to its receptors, which is a major pathway affected in people with depression (Logan, 2005).

Diets high in saturated fatty acids, such as palmitic, are considered obesogenic and promote systemic resistance to catabolic hormones such as insulin and leptin and diet-induced insulin resistance (Riccardi et al., 2004; van Dijk et al., 2009; Posey et al., 2009) whereas diets rich in monounsaturated fatty acids, such as oleic acid, attenuate weight gain through increased fat oxidation and diet-induced thermogenesis (Bergouignan et al., 2009; Obici et al., 2002; Morgan et al., 2004). Diets rich in ω-3 fatty acids promote weight loss with unaltered energy intake (Hill et al., 2007; Wang et al., 2002). This suggests that ω-3 fatty acids promote increased energy expenditure, perhaps in part by increasing fatty acid oxidation pathways and suppressing lipogenic pathways (Roche and Gibney, 1999; Sampath and Ntambi, 2005). Indeed, dysregulation of free fatty acid metabolism is responsible for insulin resistance and type 2 diabetes mellitus (Wilding, 2007) as it reduces insulin biosynthesis and secretion and induces β-cell apoptosis. As shown in this study, mice that were gavaged with either olive oil or ghee showed similar body weights during the first 6 weeks but...
ghee-gavaged mice revealed a trend of gaining more weight after the 7th week and olive oil did not induce any weight loss (Fig. 5.12). Further investigation on the role of longer-term administration of ghee and olive oil on body weight should be performed.

A consumption of a high fat, low carbohydrate diet has been widely used for weight reduction, however the increased fat consumption has been shown to raise the levels of the plasma fatty acids and this leads to impaired cognitive functions which are detrimental to the human brain (Holloway et al., 2011). According to the data in this study the quality of the dietary fatty acids in this diet can interfere with the development of mood disorders such as depression and therefore, better understanding will shed light on the best diet that will have the antiobesity effects without the negative effects on cognition and mood.

5.3.4. Chronic consumption of a diet high in palmitic acid tended to induce a depression phenotype in mice compare to olive oil

In rats that were fed isoenergetic diets based either on safflower oil (rich in ω-6 PUFAs) or beef tallow (rich in saturated fatty acids) for 8 weeks, the beef tallow group compare to the safflower oil diet group showed a significantly lower binding affinity of the β-adrenergic receptor in the hypothalamus and cortex (Matsuo and Suzuki, 1997). Moreover, the beef tallow diet decreased membrane fluidity by altering the fatty acid composition of the plasma membranes in the hypothalamus and in the cortex (Matsuo and Suzuki, 1997), suggesting that there is a possible link between membrane fluidity and the changes in affinity of β-adrenergic receptors. Moreover, dietary fat per se rather than the absolute energetic content of consumed food has been shown to promote obesity, central insulin insensitivity and hypothalamic inflammation (Posey et al., 2009) and a similar effect might happen in depression. Furthermore, the type and amount of dietary fatty acids have been shown to affect leptin receptor signalling as well as the morphology of hypothalamic cell membranes (Heshka and Jones, 2001).
Even though a positive correlation between the consumption of the western diet, which is high in many saturated fatty acids such as stearic and palmitic, and depression has been shown (Sanchez-Villegas et al., 2011), this study suggests that high levels of palmitic acid might play a role for the development of depression phenotype in mice (Fig. 5.10&5.11). As both groups showed the same increase in body weight, this suggests that the actual diet could play a direct effect on mood rather than indirectly after the development of secondary effects of obesity such as metabolic syndrome. This is in agreement with the single i.c.v. stereotactic injections of palmitic acid that suggest that we were able to induce a depression phenotype in vivo after 4 days (Fig. 5.13).

Another interesting result from this study was that the depression phenotype did not differ between groups administered with either ghee or olive oil for 3 weeks (Fig. 5.8&5.9). However, we were able to count differences in the depression levels after 7 weeks of gavage administration (Fig. 5.10&5.11). This might be due to two reasons: the first one is that the oral gavage is a very stressful process that induces high levels of stress in both tested groups therefore it was not possible to distinguish for the effect of either ghee or olive oil as they would be masked by the high stress levels of the gavage administration. Another explanation would be that high and prolonged levels of consumption of either ghee or olive oil are needed in order to see a depression effect of ghee or an antidepressant effect of olive oil.

Therefore, our data suggest the deleterious effect of palmitic fatty acid in depression and further investigation for the potential of other fatty acids that might lead to the development of depression should be studied. However, due to the fact there is no baseline in this preliminary experiment, a saline control needs to be included to check whether gavage administration alone can induce a depression phenotype in mice, and whether administration of olive oil, but not ghee, can rescue.
5.3.5. Dietary or genetic obesity induces an up-regulation of different free fatty acid receptors in the hypothalamus

The real-time PCR data showing the up-regulation of different free fatty acid receptors after dietary or genetic obesity is a very unexpected result suggesting that these receptors in the brain are affected after obesity (Fig. 5.15&5.16&5.17). Even though the role of the different fatty acid receptors has been studied extensively in different organs such as gut or pancreas, their role in the brain is totally unexplored therefore their involvement in depression remains to be clarified.

GPR41, which is a receptor for short chain fatty acids, tended to be up-regulated after the consumption of HFD and for the genetically obese (ob/ob) mice (Fig. 5.15). Acetate, a short chain fatty acid, can cross the BBB and regulate the expression profile of neuropeptides in the hypothalamus (Frost et al., 2014). Gut microbiota have been shown to produce short chain fatty acids such as acetate and release it in the circulation suggesting that it might get into the brain and regulate different pathways (Thorburn et al., 2014). The fatty acid profile analysis experiment that was performed in this study did not measure the levels of short chain fatty acids in the hypothalamus. Therefore, further investigation of whether HFD leads to accumulation of short chain fatty acids in the hypothalamus as well as investigation of the activation of the different molecular pathways under the GPR41, as is the main receptor for short chain fatty acids, in the hypothalamus should be further explored.

GPR120 is a receptor that has been linked with inflammation and insulin sensitivity (Oh et al., 2010). GPR120 RNA up-regulation was detected in the hypothalamus of the genetically obese (ob/ob) mice (Fig. 5.16). GPR120 binds mostly long chain unsaturated fatty acids, and has already been shown to act via β-arrestins (Oh et al., 2010). The role of GPR120 in the genetic mouse model of depression as well as the molecular mechanisms that it regulates in the hypothalamus needs to be investigated.
5.3.6. Up-regulation and activation of the GPR40 receptor due to dietary or genetic obesity in mice

The striking RNA up-regulation of GPR40 in the hypothalamus of mice after just 1 week on the HFD and in the genetically obese mice was a very unexpected result (Fig. 5.17). However, the protein levels of this receptor were the same between mice that consume ND and HFD (Fig. 5.18), suggesting that the actual localization of this receptor might play a role for the signalling induced by this receptor. Further studies investigating the compartmentalization of GPR40 as well as the level of activation are needed to clarify its role in the hypothalamus. GPR40 is a member of the GPCRs that has been shown to be regulated in the levels of post-translational modification as well as recycling of the receptor (Pippig et al., 1995; Krueger et al., 1997; Zhang et al., 1997; Klein et al., 2001; Gaborik and Hunyady, 2004).

The specific GPR40 agonist in vitro did not have any effect on the PDE4A5 translocation as the palmitic acid treatment did (Fig. 5.35&5.36) Previously, it has been shown that different synthetic agonists for GPR40 interact with the receptor differently than free fatty acids do (Tan et al., 2008) as long exposure of pancreas to free fatty acids impairs glucose dependent insulin stimulation (GDIS) and induce lipotoxicity whereas this effect does not happen with treatment with synthetic agonist, indicating a different mechanism of action due to binding of different ligands (Tan et al., 2008). This suggests that the GW9508 agonist regulates a different intracellular pathway than the pathway regulated by the free fatty acids via GPR40. Further investigation of the differential activation stages of GPR40 depending on the ligand should be investigated in order to determine the differential intracellular effect.
5.3.7. GPR40 antagonist administration as a therapeutic drug for the obesity induced depression phenotype

Chronic administration of the GPR40 antagonist in the hypothalamus tends to reduce the immobilization time at the depression related tests that is usually observed in mice after HFD consumption (Fig. 5.36). However, the difference was not significant and further studies with bigger cohorts of mice should be performed in order to further characterize this effect. GPR40 is a Gq-coupled GPCR that, after binding of specific free fatty acids, can induce the signalling activation of the MAPK cascade (Itoh et al., 2003), which plays an important role in inflammation (Cowan and Storey 2003). Therefore, the GPR40 antagonist might exert its beneficial effect on depression by blocking the binding of FFAs thereby blocking the activation of inflammation from contributing to the development of the depression phenotype (Raison et al., 2006; Schiepers et al., 2005). This suggests a protective role for GPR40 antagonist in inflammation and proposes that it could be used for inflammatory diseases.

Administration of the GPR40 antagonist for severely depressed patients that results from diet might present a better treatment option in comparison with invasive strategies, such as deep brain stimulations, involving high risk of infection, complicated process and high neurosurgical costs. It has already been suggested that non-invasive strategies such as receptor mediated transport (RMT), appear more attractive, because such approaches can realize transport of therapeutic agents across the whole brain without disruption of the barrier properties of the brain (Pardridge, 2002a; Pardridge, 2002b). In support of that, targeting this receptor with an antagonist might represent a good candidate for the development of an antidepressant drug.

GPR40 has been shown to play a role in both acute and chronic effects of FFAs in the pancreas (Steneberg et al., 2005), thus it is plausible that GPR40 might play a similar role in the depression phenotype of both acute and chronic effects of FFAs in the hypothalamus. As such GPR40 could have an important
role as a therapeutic target to treat depression associated with HFD. Further studies investigating a shorter and a longer time on HFD using the GPR40 antagonist should be done in order to further elucidate the effect of the blocker and obtain certainty on its effects for the treatment of depression and inflammation.

5.3.8. The phosphorylation status of GPR40 might be different between normal and depressed patients and regulated after dietary or genetic obesity

Protein phosphorylation by protein kinases and dephosphorylation by protein phosphatases represent one of the major mechanisms of signal integration in eukaryotic cells. It has already been shown that the different phosphorylation barcode of GPCRs can affect the signalling consequences of ligand-receptor interactions (Liggett, 2011; Nobles et al., 2011). This phosphorylation of GPCRs evoked by G protein-coupled receptor kinases (GRKs) and read by β-arrestins can direct different signalling cascades from the same receptor (Liggett, 2011; Nobles et al., 2011; Baillie et al., 2003). Therefore, in addition to the role of GRKs in blocking the coupling of the GPCRs with G proteins and the initiation of the desensitization signal they can also recruit additional proteins involved in receptor signalling and trafficking of the receptor (Zamah et al., 2002). GRK phosphorylation is involved in many signalling pathways and is altered during pathological situations (Penela et al., 2006). GRK phosphorylation sites have been mapped on various receptors, such as the four serine residues on the third intracellular loop, but a consensus sequence has not been established. Moreover, most cells express multiple GRKs suggesting many possible arrangements of phosphorylation between serine and threonine residues that could be phosphorylated by GRKs (Liggett, 2011; Nobles et al., 2011). However, even though many studies have examined the role of neurotransmitters and their receptors in the brain, very few have studied the involvement of different GRKs in depression and in the action of antidepressants (Garcia-Sevilla et al., 1999).
In the prefrontal cortex of people with depression who had committed suicide, the immunodensity of GRK2/3 was found to be increased, and this was interpreted as a compensation mechanism of the abnormal higher functioning of α2 adrenoreceptor (Garcia-Sevilla et al., 1999). Moreover, acute treatment of rats with desipramine (indirectly activation of adrenoreceptor by norepinephrine), but not fluoxetine, resulted in increased membrane-associated GRK2/3 in rat brain in the frontal cortex, suggesting that these receptors are regulated \textit{in vivo} by GRK2/3 (homologous regulation) (Miralles et al., 2002). Interestingly, GRK2 upregulation was observed in the membrane fraction in the prefrontal cortex of drug-free depressed subjects compared to subjects treated with antidepressants (Grangé-Midroit et al., 2003). That increase was specific for the membrane fraction, as there was no difference in the cytosol fraction (Grangé-Midroit et al., 2003), suggesting the compartmentalization of the phosphorylation is important for the action of antidepressants. GRK2 is widely expressed in the brain (Arriza et al., 1992; Grangé-Midroit et al., 2002) and it translocates from the cytosol to the plasma membrane to phosphorylate agonist activated receptor (Krupnick and Benovic, 1998; Lefkowitz, 1998; Pitcher et al., 1998). GRK2 upregulated phosphorylation of β2-adrenoreceptors is responsible for heart failure as it leads to increased Gi signalling (Zhu et al., 2012). Whether GRK2 can phosphorylate GPR40 and regulate its downstream signalling should be further studied.

Another possible mechanism of GPR40 phosphorylation in this model is by PKA. It has already been shown that PKA phosphorylates the β-adrenoreceptor and that this can affect the translocation of GRK at the membrane fraction (Li et al., 2006). This PKA phosphorylation does not affect GRK kinase activity \textit{per se}, but enhances the binding of GRK2 to the membrane and facilities its interaction with the activated receptor (Li et al., 2006).

Whether PKA can phosphorylate GPR40 and/or regulate any GRKs and β-arrestins that might interact with it needs to be evaluated. Therefore, the phosphorylation status of GPR40 by GRK2 and PKA and their interaction is
needed, as is a better understanding on the mechanisms underlying these barcode phosphorylation changes and the profile of the downstream alterations that participate in the triggering or progression of depression. These signalling consequences may contribute to the design of novel diagnostic and therapeutic strategies targeting the phosphorylation status of this receptor. The phosphorylation might have implications for drug discovery as inhibition of this phosphorylation might rescue the dietary obesity induced depression phenotype. Further investigation of the conformational states of GPR40 caused by the phosphorylation and the functional responses of this change should be done.

5.3.9. Fatty acids can modify the action of phosphodiesterases

In addition to the role of FFAs in the activation of different free fatty acid receptors, they can also enter the cell by diffusion and modify proteins in different ways such as prenylation, S-acylation, N-myristoylation. These protein modifications can affect their signalling and subcellular targeting (Sorek et al., 2009). Lipid modifications increase the hydrophobicity of proteins, facilitating their integration into intracellular and plasma membranes and it is considered a major mechanism for the compartmentalization of protein signalling (el-Husseini Ael and Bredt, 2002; Resh, 2006). Palmitoylation is a common posttranslational lipid modification that refers to the addition of palmitate to a Cys residue. The reversible nature of palmitoylation provides a potential mechanism for the shuttling of proteins between intracellular compartments (Linder and Deschenes, 2007; Fukata and Fukata, 2010). Protein palmitoylation regulates a diverse aspects of neuronal protein trafficking and function and might be implicated in various aspects of neuronal pathophysiology (Fukata and Fukata, 2010).

PDE4 enzymes can be post-translationally modified by, for example, phosphorylation (Sette et al., 1994), ubiquitination (Li et al., 2009a) and sumoylation (Li et al., 2010a). PDE4s have been shown to bind to phosphatidic acid, implying this modification is responsible for their membrane localization (Nemoz et al., 1997). PDE4D3 activation occurs through direct binding of
phosphatidic acid (Grange et al., 2000). In this study, high levels of palmitic acid were detected in the hypothalamus after the consumption of a HFD, and that correlated with the development of the depression phenotype. The increased levels of palmitic acid in the milieu could affect the activity and/or localization of the phosphodiesterases and lead to phenotypic changes. Therefore, a possible mechanism by which palmitic acid might interact with PDE4A5 would be via palmitoylation. Interestingly, using the CSS-Palm software, it was predicted that either the Cys 817 or Cys 820 might be a potential palmitoylation site on PDE4A5 (Table 5.3). The predicted score for palmitoylation was similar to the score for proteins that are known to be palmitoylated (Table 5.2). PDE10A has already been shown to be modified by palmitoylation and there is an interplay of palmitoylation and phosphorylation in the trafficking and localization of PDE10A (Charych et al., 2010). p75 neurotrophin receptor was used as it has been shown to be palmitoylated (Barker et al., 1994) to compare the prediction scores with PDE4A5.
Table 5.2. Prediction of potential palmitoylation sites of PDE4A5. CSS-Palm software was used to predict the palmitoylation scores of different cysteines on PDE4A5. Moreover, the prediction scores of two proteins known to be palmitoylated on these specific cysteines are shown (p75 and PDE10A).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Sequence</th>
<th>Sites (Aa)</th>
<th>Score</th>
</tr>
</thead>
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<td>Human</td>
<td>QIPC</td>
<td>727</td>
</tr>
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<tr>
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<td>Mouse</td>
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<tr>
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<td>279</td>
</tr>
<tr>
<td>PDE10A</td>
<td>NP_035996</td>
<td>Mouse</td>
<td>NASCFRR</td>
<td>11</td>
</tr>
</tbody>
</table>
5.3.10. *In vitro* fatty acid treatment of neuronal cells expressing GPR40

In this study, the differential effect of palmitic versus oleic acid on the translocation of PDE4A5 at the membrane fraction was observed (Fig. 5.28 & 5.30). This data is in agreement with another study showed that in the hypothalamus palmitic but not oleic acid induces the translocation of PKC-θ at the membrane fraction (Benoit et al., 2009).

During the *in vitro* treatments of the cells with the fatty acids in this study, a great deal of variability was observed between experiments concerning the stimulation stage of cells. Different fetal bovine serum consists of differential amount of fatty acids, and this may contribute to the observed variability. More experiments should be performed to identify the exact experimental conditions with the correct time and concentration of fatty acids in order to see stimulations of GPR40 and the effects on PDE4A5 and arrestin translocation. In accordance with these observations, it has been shown previously that GPR40 is a receptor that is highly activated under normal conditions due to endogenous ligands, and these high levels of activation were reversed after addition of BSA free fatty acid in the media that traps the fatty acids that exists in the media from the serum (Stoddart et al., 2007). Thus to perform *in vitro* stimulation of GPR40, we need to deprive the medium of GPR40 endogenous ligands.

Another caveat to keep in mind during *in vitro* treatments with fatty acids is the fact that long chain fatty acids are relatively insoluble in aqueous solution and form micelles at higher concentrations that can bind to plastics and glassware. This can greatly reduce the active unbound monomeric concentration of the fatty acid, in a manner analogous to the effects of albumin. On the contrary, short-chain saturated fatty acids, such as butyric acid, as well as long chain unsaturated fatty acids such as oleic acid, are water soluble in aqueous solution. It is possible that these differences might had an effect in the experimental system used in this study.
Lastly, fatty acids have been shown to exert a detergent effect on cell membranes, possibly via FFA exposure of GPR40 (Steneberg et al., 2005). However, the concentrations used in this study are well below those at which fatty acids exert detergent effects on cell membranes (Cistola et al., 1988).

### 5.3.11. The central role of GPR40 in the regulation of neurogenesis is signalled by fatty acids in the hypothalamus

A major characteristic in depressed patients is reduced neurogenesis (Snyder et al., 2011) (Eisch and Petrik, 2012). Of the various molecules that contribute to adult neurogenesis, as well as depression, the most important is cAMP response element-binding protein (CREB), a transcription factor which is a rapidly responding intracellular effector of signalling pathways in neurons (Carlezon et al., 2005). Reduction in CREB activation leads to a depression phenotype, whereas global inhibition of degradation of the cAMP pathway results in an antidepressant effect (Nibuya et al., 1996a). The cellular localization of pCREB is identical to that of GPR40 in the hippocampal neurogenic niche, and both are upregulated after ischemia, which functionally links them both in hippocampal neurogenesis (Ma et al., 2008a; Boneva and Yamashima, 2012). This is suggested to happen after the binding of PUFAs on GPR40, and might trigger the phosphorylation of CREB, which and that might result in specific gene activation - intracellular events that are crucial for adult neurogenesis, cognition and depression.

The influx of different fatty acids in the hypothalamus after the consumption of the HFD observed in this study (Fig. 5.3) might play a central role in the signalling of neurogenesis or apoptosis. Palmitic or stearic acid can induce apoptosis in neuronal cells (Uulloth et al., 2003) that can be prevented by treatment with unsaturated fatty acids (Almaguel et al., 2009; Welters et al., 2006). Moreover, different fatty acids have been shown to have opposing effects on food intake or body weight in the hypothalamus (Schwinkendorf et al., 2011), suggesting that mere infusion of fatty acids is not responsible, but rather distinct signals involving specific FA are most likely involved.
Furthermore, in this study it has been shown that HFD consumption increases RNA levels of GPR40 (Fig. 5.17). As a large range of both saturated and unsaturated fatty acids are ligands for GPR40, the primary role of the receptor may be to distinguish the ligand. Different ligands will lead to the initiation of different intracellular pathways and yield different result. This might explain how palmitic acid can cause the development of the depression phenotype while unsaturated fatty acids have a protective effect (Mozaffarian et al., 2010). Binding of palmitic acid on GPR40 might lead to apoptosis whereas unsaturated fatty acids might play a protective role and induce neurogenesis. Moreover it has been shown that short or long exposure of fatty acids to GPR40 has a differential effect in the pancreas (Steneberg et al., 2005), suggesting that the length of the HFD consumption might play a role in the development of depression phenotype. The differential role of the different fatty acids, such as oleic or palmitic, should be explored, including their different downstream effects of either neurogenesis or apoptosis, which can potentiate depression.

The role of nutritional factors in brain cognition and behaviour is a new area of research. The direct and differential contribution of dietary fatty acids in the context of mental depression, focusing on dietary experimental paradigms, deserves further investigation. Although the agonist action on cell surface receptors can trigger a variety of intracellular signalling events, the ligand-receptor interaction and their downstream responses are very specific. The potential protective effects of polyunsaturated fatty acids, antioxidant micronutrients, and folates against age-associated cognitive and mood disorders due to a diet high in trans and saturated fat are arousing increasing interest. In further studies towards the understanding of the mechanism of depression due to dietary factors and its therapeutic implications for medicine, it will be indispensable to comprehend the molecular interactions within this complex process that precede the development of depression.
5.3.12. The potential sequence of the interaction between PDE4A5/ARB2/GPR40

It has been shown that GPCRs can directly or indirectly recruit certain phosphodiesterases via the help of scaffolding proteins such as β-arrestins (Houslay, 2010; Perry et al., 2002). This interaction has been described clearly with peptide arrays and mutagenesis studies (Baillie et al., 2007). This study is the first to show an interaction of a free fatty acid receptor with PDE4A5.

Several reports have described the significance of a highly conserved stretch of amino acid residues located in the second intracellular loop of various GPCRs (Glu/Asp)-Arg-Tyr in G protein activation (Probst et al., 1992; Savarese and Fraser, 1992). This domain is generally designated the DRY motif and is considered to maintain the receptor conformation (Ballesteros et al., 1998; Ballesteros et al., 2001). In particular, the central Arg residue of this motif has been reported to be directly responsible for the receptor G protein coupling. This motif has also been involved in the binding of β-arrestins. **Figure 5.38** shows a structural alignment of GPR40 with β-adrenoceptor using the T-coffee online algorithm (Chang et al., 2012). GPR40 has the GRY motif instead of DRY. The importance of this change and how it affects the binding of G proteins or arrestins should be further investigated.
Figure 5.38. Structural alignment of human and mouse GPR40 with beta 2 adrenergic receptor. The multiple sequence alignment result was produced by TM-Coffee, Version_9.02.r1228 (2012-02-16 18:15:12 - Revision 1228 - Build 336).

From the structural analysis, the suggested intracellular loops as well as the C-terminal tail of GPR40 are:

c1: SHAKRLTP

c2: LGAAFPFGYQAIRRP

c3: VGCLRALVRSGLSHKRKL

c-terminal: GYLGTGPGRTICVTRTGRTIQK

Further characterization of the interaction of ARB2 with GPR40 should be studied via peptide arrays and mutation analysis. Moreover, the potential serines or threonines on the intracellular loops that might be phosphorylated by GRK or PKA should be studied.
5.4. Conclusions

The clinical and epidemiological data point in the same direction as our current findings and strongly support a causative role of the western dietary habits on depression. We discovered that a diet high in saturated fatty acids can markedly increase the influx of different fatty acids in the hypothalamic area of mice. The different free fatty acid receptors are in the unique position to link food lipids and mood disorders such as depression. Levels of GPR40 are markedly increased in the hypothalamus after dietary or genetic obesity, and this novel finding was also accompanied by a partial rescue of dietary obesity induced depression after GPR40 antagonist administration. These data provide a potential mechanism through which dietary or genetic obesity can play a causative role for the development of depression phenotype. Moreover, administration of the GPR40 antagonist might be a better way of treating depression compared to other invasive strategies that involve high risk of infection, complicated and high neurosurgical costs. Further studies on this topic are needed to assess the potential of GPR40 antagonist as an antidepressant. However, even though fatty acids can act as signalling molecules, very little is known about the mechanisms of saturated fatty acid function in mental disorders. The exact molecular mechanism by which saturated fatty acids are induces depression should be further investigated.
Chapter 6

Final Discussion
6. Background

Depression is a psychiatric disorder associated with excessive sadness and other alterations of mood, cognition and neurovegetative functions such as sleep and appetite. It is a leading cause for long term disability worldwide and, as such, carries a considerable human and economic cost (Moussavi et al., 2007). The cAMP signalling cascade provides a central facet in the regulation of signalling pathways involved in depression, being a downstream effector of both the serotonin and noradrenergic GPCRs (Shelton, 2007). In particular, different antidepressant treatments have been shown to alter the levels of protein molecules involved in the cAMP signalling cascade (Duman et al., 1997).

The relationship between obesity and depression has also been widely discussed, as many epidemiological studies have consistently identified a strong positive association between depression and obesity (Faith et al., 2002). However, epidemiological and clinical studies are unable to provide insight in the causative relation and mechanistic linkages associated with this relationship due to other life style factors in humans that can interfere. My PhD project was aimed to provide mechanistic insight into the putative link between obesity and depression using two different models of obesity.

Within this body of work I have utilised in vivo models to investigate the role of obesity in depression in mice as well as in vitro methods in order to elucidate the molecular pathway that may link obesity with depression. Several postulates were evaluated with regard to establishing the molecular and behavioural link between obesity and depression. First of all, I examined whether obesity, either dietary or genetic can be a causative factor for the development of depression. Secondly, as to what are the signalling cascades that are being affected after the consumption of a diet high in saturated fatty acids. Thirdly, I wished to establish what is/are the key molecule/s that play/s a pivotal role linking obesity with depression and the intracellular molecular mechanism that links diet with mood disorders such as depression. While, the fourth and final aim was to try and identify a novel therapeutic target for depression that could
potentially be used to treat obese and overweight populations that are resistant to other antidepressant drugs.

### 6.1. Obesity is a causative factor for the depression

Previous epidemiological studies have revealed a link between obesity and depression (Faith et al., 2002). However there has been no causal link identified between the two and no indication on the mechanism. A major challenge in exploring the relationship between diet and depression is due to the complexity of dietary, environmental and lifestyle parameters. Thus there is a need to dissociate or control these factors in order to actually detect causality in human studies. Moreover, some characteristics, such as paucity of exercise and obesity, usually pair together and are difficult to dissociate. Therefore, animal models are often employed to control such heterogeneity and the many variables inherent to human populations. Animal models provide a more homogeneous environment and background and are easier to study and evaluate. Using behavioural paradigms I established that obesity, either dietary or genetic, can be a causative factor for the development of depression (Fig.3.2&3.3&3.8). Such an effect is independent of the increase in body weight caused by diet (Fig.3.5). I also demonstrated that dietary obesity does not induce anxiety, suggesting that the depression phenotype observed in mice is independent of stress inducers (Fig.3.10).

### 6.2. Dietary obesity down-regulates the cAMP/PKA signalling pathway in the hypothalamus

Chronic antidepressant treatment regulates the levels of adenylyl cyclase and PKA in the brain (Menkes et al., 1983; Nestler et al., 1989b; Perez et al., 1989; Ozawa and Rasenick, 1991). Fibroblasts from patients with major depression exhibit significantly less PKA activity linked with β-adrenoreceptor function than do normal subjects, even though cAMP is able to activate PKA similarly in both groups (Shelton et al., 1996). Both antidepressant treatment and electroconvulsive seizure therapy have been shown to reduce cytosolic PKA activity while increasing nuclear PKA localization in rat frontal cortex (Nestler et al., 1989a). Therefore, a link between antidepressant drugs, electroconvulsive
seizures and PKA functioning has been established. However the molecular mechanism as well the gene targets involved in such PKA signalling are unknown.

Depression is in many ways a “pleiotropic” disorder that involves disturbances in mood, sleep, autonomic, endocrine, appetite, sexual, immune and other functions. Protein kinase A (PKA) exerts “pleiotrophic” actions by linking a variety of receptors and affecting, by means of protein phosphorylation, enzymes involved in neurotransmitter synthesis, regulation of neurotransmitter receptors, ion channels, enzymes involved in the regulation of second messengers, transcription factors such as cAMP responsive element modulator protein CREB, proteins involved in both DNA transcription and mRNA translation as well as the cytoskeleton. Protein phosphorylation by PKA has been proposed as a critical pathway in the regulation of different neuronal functions (Kandel, 2012). Therefore, given the wide range of PKA targets this might be the reason of the wide range of abnormal cellular responses that the depressed patients exhibit (Shelton et al., 1996).

The mRNA array analysis of hypothalamic samples of mice fed HFD compare to ND revealed a decrease in the RNA of many molecules involved in the PKA signalling cascade (Fig.3.11&3.12). Gratifyingly, these data were in agreement with the western blot data that also highlighted the decrease in levels of PKA phosphorylation of hypothalamic proteins in animals fed HFD compare to controls (Fig.3.13). However, the mRNA array analysis did not reveal any RNA changes of the PDE4A, CREB or GPR40 (Fig.3.11&3.12) that have been examined in this study suggesting that the effect of these genes on depression might synergically depend on other signalling molecules involved in this phenomenon.

PKA provides a central effector for the cAMP signalling pathway whose activity is directly controlled by the levels of cAMP. cAMP binding to the regulatory (R) subunit of PKA leads to the activation of the PKA catalytic (C) subunit that is now able to phosphorylate associated protein substrate targets. cAMP signalling has been shown to be down-regulated in depressed patients (Mazzola-Pomietto et al., 1994). Moreover, many antidepressant drugs act via the upregulation of molecules at the cAMP signalling which is the major regulator of PKA (Duman et
A similar mechanism of action suggesting the upregulation of the cAMP signalling pathway has been proposed for rolipram, a specific phosphodiesterase4 inhibitor with antidepressant action (Zeller et al., 1984; Kehr W, 1985; Overstreet et al., 1989). In accordance, this study revealed that the down-regulation of PKA signalling when analysed by mRNA array after HFD consumption (Fig.3.11&3.12&3.13) was accompanied by the down-regulation at the total levels of cAMP in the hypothalamus in vivo (Fig.3.14). Taken all together these data reveal for the first time that HFD induces a down-regulation of the cAMP/PKA signalling cascade in the hypothalamus and this is implicated behaviourally for the development of depression in mice.

6.3. The central role of PDE4A5 for the dietary or genetic obesity induced depression phenotype

Cyclic nucleotide phosphodiesterases (PDEs) are enzymes that provide the sole route for cAMP degradation in cells (Conti and Beavo, 2007; Bender and Beavo, 2006; Maurice et al., 2003). PDEs are a big family with each member displaying distinct roles and intracellular localization (Houslay et al., 2007; Lugnier, 2006; Conti and Beavo, 2007). Rolipram, a generic PDE4 inhibitor, has been shown to have antidepressant action in mice and humans (Zeller et al., 1984; Kehr W, 1985; Overstreet et al., 1989). However, despite rolipram’s promising antidepressant actions in mice and man, it causes significant side effects owing to its mechanism of action as a non-isoform-specific PDE4 inhibitor. This made it impossible to develop as a human therapeutic (Dyke and Montana, 2002; Robichaud et al., 2002; Hirose et al., 2007; Bertolino et al., 1988; Hebenstreit et al., 1989). PDE4 enzymes are major regulators of the cAMP signalling in the brain (Jin et al., 1999) and have been shown to localize in brain regions that are associated with reinforcement, movement and affect, all of which are affected in people with depression (Cherry and Davis, 1999).

Chronic, but not acute, rolipram treatment in mice, has been shown to have an antidepressant effect in humans and mice (Tardito et al., 2006; Przegalinski et al., 1985). This is in accordance with our study that revealed that only chronic administration tends to reduce the depression phenotype due to the consumption of the HFD (Fig.4.2&4.3) implicating the role of the PDE4/cAMP
pathway. This experiment was accompanied with the unexpected observation that when rolipram was injected into mice they did not gain any body weight even on HFD. In contrast to this, Prozac injected mice gained significantly more body weight even from the first administration (Fig.4.4&4.5). The resistance to body weight gain of the mice that were daily injected with rolipram even though they were on a HFD might be explained by the fact that rolipram has been suggested to alter metabolic processes in adipose tissue (Doseyici et al., 2014) whereas the role of the serotonin signalling under HFD conditions should be further investigated.

Next, my aim was to investigate the role of PDE4s in depression due to obesity. With this in mind the cAMP PDE4 activity in different brain regions was investigated after the consumption of HFD. An increased PDE4 activity was detected specifically in the hypothalamus of mice fed HFD compare to ND (Fig.4.7). Associated with this we observed an increase in total protein and phosphorylation state of PDE4A5 in the hypothalamus of mice fed HFD compare to ND (Fig.4.14&4.21). Identifying the specific isoform responsible for the antidepressant action of rolipram may be of potential therapeutic importance as the design of a specific inhibitor will minimize the side effects that a generic PDE4 inhibitor poses. Comparative real time PCR analysis revealed a specific increase of PDE4A and, specifically, for the PDE4A5 isoform at the RNA levels in the hypothalamus of mice fed HFD compare to ND (Fig.4.13) suggesting that the specific inhibition of PDE4A5 might lead to the rescue of the depression phenotype due to obesity.

The role of the transcription factor CREB in depression is very controversial as some studies suggest the increase or the decrease phosphorylation of CREB being important component for the action of the antidepressant treatment (Carlezon et al., 2005). Moreover, it has been suggested that the regulation of CREB might also be cell-type or brain region specific. CREB is directly phosphorylated by PKA and can regulate the transcription of a well-defined and substantial cohort of genes. As the total phosphorylation levels of PKA were down-regulated after HFD (Fig.3.12) the total phosphorylation levels of CREB was tested. Western blot
analysis revealed down-regulation of total p-CREB levels of in the hypothalamus of mice fed HFD compare to ND (Fig.4.15).

Another target of the cAMP signalling cascade is the neurotrophin factor, BDNF that has been extensively studied for its role in depression (Pittenger and Duman, 2008). BDNF expression in the hippocampus is down-regulated after HFD in rodents (Molteni et al., 2002) and this is associated with major depressive disorder (Yulug et al., 2009). However, in our model of depression we did not detect any BDNF RNA changes after the dietary or genetic obesity in the hypothalamus (Fig.4.30).

Another direct target for PKA phosphorylation is DARPP-32, a protein associated with the dopamine receptor (Walaas and Greengard, 1984) that has been involved in depression (Svenningsson et al., 2002). Western blot analysis data for the total levels of DARPP-32 phosphorylation revealed a trend for an increase after the consumption of the HFD (Fig.4.31). Increased phosphorylation of DARPP-32 by PKA was observed even though there was a global down-regulation of the PKA phosphorylation. This suggests that DARPP-32 lies in a cellular sub-compartment where PKA is still highly active; indicating perhaps that compartmentalized PDE activity in such a localization may not be increased or even decreased so as to allow this.

6.4. The loss of PDE4A in vivo was able to rescue the dietary and genetic obesity induced depression phenotype

Given the biochemical data supporting an important role of PDE4A5 in depression a collaboration was established with Professor Marco Conti, UCSF to obtain genetic knockout PDE4A<sup>−/−</sup> mice in order to investigate the in vivo loss of PDE4A gene. The PDE4A<sup>−/−</sup> as well as the PDE4A<sup>+/+</sup> mice were fed HFD and tested with behavioural paradigms. The double knock out ob/ob:PDE4A<sup>−/−</sup> was also generated in order to check the effect of the loss of PDE4A in a genetic mouse model of obesity. Interestingly, the loss of PDE4A gene in vivo was able to rescue the dietary and genetic obesity induced depression phenotype (Fig.4.16). This effect was independent of the body weight gain, as both PDE4A<sup>−/−</sup> and PDE4A<sup>+/+</sup>
mice on HFD gain the same body weight as well as \textit{ob/ob} and \textit{ob/ob:PDE4A^{+/−}} on ND (Fig.4.17&4.18). Previously, I have described that HFD increases the activity of PDE4 in the hypothalamus (Fig.4.7). Moreover, through separation of the cytosol from the membrane fraction I was able to identify that the specific increase in PDE4 activity was associated with the membrane fraction (Fig.4.19). This increase of the PDE4 activity correlates with the increased level of PDE4A5 phosphorylation (Fig.4.21) and is in agreement with previous studies showing a positive correlation between increased phosphorylation of PDE4A5 and its enzymatic activity (Laliberte et al., 2002). This is in accordance with previous studies that PDE4 isoforms are targeted and interact with specific proteins/lipids in cells (Houslay and Adams, 2003) and in so doing they play a pivotal role in underpinning the compartmentalization of cAMP signalling (Mongillo et al., 2004). That increased PDE4 activity at the membrane fraction was abolished for the PDE4A^{−/−} mice compared to the PDE4A^{+/−} mice, which suggests no compensatory mechanisms from the other isoforms and further suggests a specific role of PDE4A in this locality (Fig.4.19). However, the \textit{ob/ob} and the \textit{ob/ob:PDE4A^{+/−}} mice showed a different pattern of PDE4 activity as there was an increased PDE4 activity at the cytosol fraction of hypothalamic samples as well as the membrane fraction of \textit{ob/ob:PDE4A^{−/−}} compared to \textit{ob/ob} (Fig.4.22&4.23) suggesting another mechanism involved for the genetic obesity induced depression phenotype. These PDE4 activity measurements for the \textit{ob/ob} and \textit{ob/ob:PDE4A^{−/−}} are opposite observations from the dietary obesity induce depression phenotype and suggest that in this genetic mouse model of obesity there might be a compensatory mechanism for the loss of PDE4A in the PDE4A^{−/−} knockout mice on an \textit{ob/ob} background. Further investigation into the exact role of PDE4 in the leptin deficient \textit{ob/ob} mice is needed. Nonetheless, the data presented regarding PDE4 activity shows for the first time the role of PDE4 activity in models of dietary and genetic obesity.
6.5. Dietary or genetic obesity induced depression was not caused due to cytokine expression

A “cytokine hypothesis” of depression is supported by the observation that depressed individuals have elevated plasma levels of certain cytokines compared to healthy controls (Schiepers et al., 2005). Systemic low grade inflammation is a consistent finding in metabolic disorders (Hotamisligil, 2006) and the resulting elevated level of circulating cytokines is suggested to promote depression (Raison et al., 2006). Depression has been linked with peripheral and central inflammation and increase of cytokines such as TNF-α, IL-1 and IL-6 in peripheral blood and cerebrospinal fluid (Watkins et al., 2014; Logan, 2003). All these molecules have been implicated in obesity as well (Park et al., 2010a). An altered HPA-axis is often seen in depressed individuals and may be involved in the development of metabolic syndrome (Turnbull and Rivier, 1995). In the light of this, I set out to evaluate transcript levels of different cytokines in the hypothalamus of our model of depression. Interestingly, there was no increase in cytokine expression in the hypothalamus of mice fed HFD and for the genetically obese mice, suggesting the exclusion of the involvement of these molecules in this model of depression (Fig.4.26&4.27&4.28). The real time PCR data are in accordance to the mRNA array analysis data done in this study as the inflammatory pathways were not affected after the consumption of the HFD shown by the array analysis by ingenuity.

6.6. PDE4A5 overexpression in the hypothalamus induces hyperphagia and obesity at the PDE4A−/− mouse

Depression has been linked with appetite disorders as people with depression are characterized by irregular eating habits. Despite the significant advances in the understanding of the molecular mechanism of appetite and satiety (Wilding, 2002; Schwartz et al., 2000; Woods and D'Alessio, 2008) practical therapies for weight loss or appetite disorders remain elusive. Central control of food intake is highly involved in emerging metabolism and body weight regulation (Lee and Wardlaw, 2007; Cota et al., 2007; Moran, 2010). Given the rising rates of obesity, this has inspired efforts to understand the brain regulation of food intake and energy expenditure. The main regulators of appetite are insulin and
leptin. Leptin is secreted from white adipose tissue (WAT) and insulin is secreted from pancreatic β cells. These hormones, can cross the BBB, and get access in the brain to signal directly at their receptors in the ARC, ventromedial hypothalamic nucleus (VMN), NTS and elsewhere (Grill and Hayes, 2012; Schwartz et al., 2000) in order to regulate different pathways. Both leptin and insulin exert their effects on energy balance in part by activating the hypothalamic melanocortin system (Benoit et al., 2002; Seeley et al., 1997). The hypothalamic melanocortin system has a predominant role in food intake regulation and energy homeostasis (Adan et al., 2006; Seeley et al., 2004; Vergoni and Bertolini, 2000). This system consists of first order pro-opiomelanocortin (POMC) and agouti-related peptide (AgRP) expressing neurons in the ARC that project to melanocortin-4 receptor (MC4R)-positive neurons that exist in the paraventricular nucleus of the hypothalamus (PVN), the lateral hypothalamic area (LHA) and other brain regions (Cone, 2005). This system consists of heterogeneous sets of neurons primarily in the arcuate nucleus expressing various orexigenic and anorexigenic neuropeptides (Lee and Wardlaw, 2007). Orexigenic neuropeptides such as neuropeptide Y (NPY) and agouti related protein as well as anorexigenic neuropeptides such as those derived from pro-opiomelanocortin (POMC) act through multiple mechanism to control energy homeostasis (Chee and Colmers, 2008; Coll et al., 2005; Ilnytska and Argyropoulos, 2008). Interestingly it has been shown that adeno-associated virus-mediated knockdown of melanocortin-4 receptor in the paraventricular nucleus of the hypothalamus promotes high-fat diet-induced hyperphagia and obesity (Garza et al., 2008). The role of the cAMP pathway in the melanocortin, as well as the leptin system, and whether cAMP regulates orexigenic and anorexigenic peptide production, is unknown. However, brain leptin and insulin signalling activate POMC and inhibit AgRP neurons (Cowley et al., 2001; van den Top et al., 2004). The net effect of this is to increase MC4R signalling and, thereby, to reduce food intake and increase energy expenditure.

Here we show, for the first time, that the introduction of PDE4A5, via viral overexpression in the hypothalamic area, in vivo can promote hyperphagia and obesity in mice (Fig. 4.37). These data involve the role of the cAMP signalling pathway as increased PDE activity in the hypothalamus results in the reduction
of cAMP signalling. The extreme increase in body weight of these animals injected with the PDE4A5-wt virus and subjected to three weeks on HFD is of particular significance. Interestingly, another study recently showed that the leptin effect on feeding depends on a pathway regulated by phosphatidylinositol-3 kinase and PDE3B in the hypothalamus (Zhao et al., 2002). The identification of the hypothalamic nuclei involved in this phenomenon as well as the molecular mechanism remains to be found.

Therefore, further studies on the role of PDE4A5 as a potential regulator of the appetite in the hypothalamus and its role on the neuropeptide expression affecting food intake and body weight should be performed. Based on our observations, targeting components of the cAMP signalling such as PDE4s might represent a good target for the treatment of eating disorders. Food intake targeting would be of particular interest, considering the recent rejection of rimonabant for the treatment of obesity.

6.7. **Specific PDE4A5 down-regulation in the hypothalamus might lead to the rescue of dietary or genetic obesity induced depression phenotype as well as treat appetite disorders**

Further investigation of the specific role of PDE4A5 on the link between obesity and depression as well as its role in appetite is needed. The creation of a specific PDE4A5 inhibitor will shed light on the exact role of this isoform in the brain and will dissociate its action and side effects from the other PDE4 isoforms. The creation of a specific inhibitor from the PDE4 family to be used as antidepressant is very important as it might be used in combination with current antidepressant treatment for faster antidepressant results as repeated co-administration of rolipram with antidepressants has been shown to show faster antidepressant effects than antidepressants alone (Fujimaki et al., 2000).
Recently, antisense oligonucleotide therapy has been used in order to down-regulate the mRNA expression of specific genes (Kordasiewicz et al., 2012). The Food and Drug Administration (FDA) has already approved the antisense oligonucleotide therapy for homozygous familiar hypercholesterolaemia. Sequence alignment between the different PDE4A isoforms revealed a specific area on the PDE4A5 for effective and specific targeting. Using this area 7 candidate 21 base antisense oligonucleotide sequences were designed. Homology analyses were conducted for each oligonucleotide using the rat genome database to ensure that the sequence is unique to the mouse PDE4A5 gene. Further testing of these sequences which one can specifically down-regulate the PDE4A5 is needed. Table 6.1 shows the proposed sequences of the antisense oligonucleotides that should be further tested. Figure 6.1 shows a sequence alignment of the mouse PDE4A5 with the antisense oligonucleotides. Specific down-regulation of PDE4A5 might be an interesting means for treating obesity induced depression as well as for targeting eating disorders.

**Table 6.1. Proposed sequences for the downregulation of PDE4A5.** Sequences of the 7 proposed antisense oligonucleotides that might downregulate the expression of PDE4A5 specifically.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOs1</td>
<td>GAGACAGGCTCCTCTCCGAG</td>
</tr>
<tr>
<td>AOs2</td>
<td>AAGAGAGAGAGACAGGCTCC</td>
</tr>
<tr>
<td>AOs3</td>
<td>GTAGCCCGCCTGCTGGATGC</td>
</tr>
<tr>
<td>AOs4</td>
<td>CTGTCCGAGTAGCAGGCTCT</td>
</tr>
<tr>
<td>AOs5</td>
<td>TGAGCGCTCGGACTGTCCCG</td>
</tr>
<tr>
<td>AOs6</td>
<td>CAGGACATGCAGGTAGCCCG</td>
</tr>
<tr>
<td>AOs7</td>
<td>ACCGGGTGCCGTGGAAGGACG</td>
</tr>
</tbody>
</table>
Figure 6.1. Sequence alignment between the proposed antisense oligonucleotides and the mouse PDE4A5. The sequences of the 7 proposed antisense oligonucleotides that might downregulate the expression of PDE4A5 specifically alignment with mouse PDE4A5 is shown.

6.8. The effect of the influx of dietary fatty acids in the hypothalamus

The effect of consumption of a HFD has been studied in relation to metabolic syndrome and inflammation (Hwang et al., 2010). However, what is the exact nature of the pathway that the fatty acids follow when they enter our body via the diet is unknown. Further studies using labelled fatty acids in the diet and studying their accumulation in the body are needed. Obesity is a condition characterized by high levels of circulating fatty acids suggesting a detrimental role of these fatty acids in the human physiology of overweight or obese individuals (Carpentier et al., 2000; Sako and Grill, 1990). Chronic physiological excess of FFAs can induce the dysregulation of physiological processes as it has been shown to pancreatic function (Steneberg et al., 2005). In this study, fatty acid profile analysis in the hypothalamic area of mice fed HFD revealed an
increase influx of saturated and unsaturated fatty acids in the hypothalamus (Fig. 5.5). The hypothalamus is a highly vascularized brain region with a lot of communication with the periphery as it orchestrates the energy balance centrally with the periphery. Nutrient sensing within the hypothalamus has a critical role in the complex network of signals controlling energy metabolism (Cota et al., 2007; Le Foll et al., 2009; Moran, 2010). Recently, lipid signalling in the brain has been shown to play an important role in regulating energy balance (Lam et al., 2005b; Lopez et al., 2005). The accumulation of the different fatty acids in the hypothalamus shown in this study and the molecular pathways they regulate in energy balance as well as mood disorders in the brain regions should be further studied. Saturated fatty acids alter the membrane distribution of proteins such as Src that are responsible for the activation of inflammatory signals and cause their partitioning into intracellular membrane subdomains in order to be activated whereas unsaturated fatty acids prevent that effect (Holzer et al., 2011). Even though the incorporation of fatty acids can alter the fatty acid composition at the plasma membrane resulting in altering the structure and function by changing membrane fluidity and signal capacity (Clamp et al., 1997; Karnovsky et al., 1982; Luo et al., 1996) the mechanism responsible for discriminating between saturated and unsaturated fatty acids and how this effect is responsible for the induction of mood disorders such as depression is unknown.

6.9. The potential role of palmitic acid for the development of depression

Consumption of western diet that is high in saturated and trans fat has been linked with depression. However, diets rich in specific fatty acids have only been studied in relation to obesity and energy balance. Diets rich in saturated fatty acids, such as palmitic acid, are considered obesogenic and promote systemic resistance to catabolic hormones such as insulin and leptin (Riccardi et al., 2004; van Dijk et al., 2009). On the contrary, diets rich in monounsaturated fatty acids, such as oleic acid, attenuate weight gain through increased fat oxidation and diet-induced thermogenesis (Bergouignan et al., 2009) have a protective role and can reverse the obesity induced insulin resistance (Robinson et al., 2007). However, diets rich in specific fatty acids and the specific dietary
components that lead to the induction of depression, as well as the molecular mechanisms of this phenomenon have never been studied. In this study it was shown for the first time that ghee, which is high in palmitic acid, was able to partially induce a depression phenotype \textit{in vivo} compare to a diet with olive oil that is high in oleic acid, an unsaturated fatty acid (Fig. 5.12 & 5.13). To be able to demonstrate a depressant effect mice had to be fed with either ghee or olive oil by oral gavage for long period of time (7 weeks) as the 3 week time point that was tested did not show any difference (Fig. 5.10 & 5.11). These data are in agreement with the preliminary data of the bilateral single stereotactinic injections of palmitic acid in the hypothalamus that the mice developed a depression phenotype (Fig. 5.15). Future studies will establish whether palmitic acid plays a role as a causal factor in depression \textit{in vivo}. It is possible that palmitic acid derived from food can cross the BBB and act directly in the hypothalamus to suppress the cAMP pathway through central hypothalamic mechanisms in order to induce a depression phenotype.

Interestingly, two recent human studies suggest that a reduced caloric intake from fat and processed food is associated with improvements in mood and reduced risk of developing depression (Akbaraly et al., 2009; Brinkworth et al., 2009), which agrees with our findings. The nutritional epidemiology of depression probably deserves a higher attention because it is one of the most relevant single contributors to the global burden of disease, being the world leading cause of years of life lived with disability for both men and women (Lopez et al., 2006).

6.10. Upregulation of the RNA levels for the different free fatty acid receptors in the hypothalamus of mice fed HFD

Even though epidemiological and clinical studies have shown a link between western diet and depression the mechanism/s of this phenomenon is totally unknown. Free fatty acid receptors have been studied extensively for their role in the gut and in pancreas (Itoh et al., 2003; Thorburn et al., 2014). However, their role in the brain remains unknown. The free fatty acid receptors are in the unique position to explain how dietary fatty acids can link food with mood...
disorders such as depression. The finding of the RNA upregulation of the different free fatty acid receptors in the hypothalamus after dietary and genetic obesity is a very interesting result (Fig. 5.17 & 5.18 & 5.19). More strikingly, the 4-5 fold increase at the RNA levels of GPR40 even after 1 week on HFD involves GPR40 in the regulation of different signalling in the hypothalamus and more specifically in depression (Fig. 5.19). Therefore, for the first time, a free fatty acid receptor has been implicated as playing a role in depression.

6.11. Palmitic but not oleic acid alters the activation of the PKA signalling cascade

The role of macronutrients such as lipids in the control of metabolism and food intake in the hypothalamic nuclei have been studied extensively and how they regulate the circulating levels of leptin and insulin (Woods et al., 1998) which in turn modulate appetite, energy expenditure, and intermediary metabolism mainly via their hypothalamic receptors nuclei (Woods et al., 1998) (Schwartz et al., 2000). Different lipids differentially regulate orectic and anorectic effects as well as apoptosis (Welters et al., 2006) (Newsholme et al., 2007) as saturated fatty acids have been shown to produce an orectic effect whereas unsaturated fatty acids such as oleic an anorectic (Obici et al., 2002) (Milanski et al., 2009). Palmitate has been shown to have a strong apoptotic effect whereas monounsaturated fatty acids have a protective effect in the induction of apoptosis (Newsholme et al., 2007) (Maedler et al., 2003). Moreover, saturated but not mono-unsaturated fatty acids induce apoptotic cell death in neonatal rat ventricular myocyte (de Vries et al., 1997). Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4 (Lee et al., 2001). Saturated and unsaturated fatty acids have been shown to have a different effect on beta-cell apoptosis (Eitel et al., 2002).

However, how lipids regulate intracellular signalling pathways to have this effect and whether they can influence the PKA signalling cascade that has been involved in our model of depression has never been studied. A mouse neuronal cell line was used in this study has been shown to express high levels of the GPR40 receptor. GPR40 has been shown to bind a wide range of fatty acids such
as oleic, palmitic and myristic (Briscoe et al., 2003). Neuronal cells transfected with a PKA sensor and treated with forskolin and IBMX demonstrated an increase at the FRET signal (Fig. 5.7). However, neuronal cells pretreated with palmitic acid were unable to show the forskolin induced activation of the PKA sensor (Fig. 5.7). On the contrary to this, neither myristic nor oleic acid pretreatment of cells transfected with the same PKA sensor inhibited the activation of PKA by forskolin (Fig. 5.7). The differential effect of these different fatty acids suggests their differential role on the PKA signalling cascade. Thus binding of palmitic acid to GPR40 can provide a signal for reduction of cAMP signalling that in turn activates a chain of neuronal events designed to promote depression. Even though in our study we focused on the role of oleic, palmitic and myristic acids, it still remains to be determined whether other fatty acids have a similar effect.

6.12. Targeting the GPR40 receptor as a new molecule for the treatment of depression caused by diet

Considerable focus has been placed on developing agents targeting the serotonin or noradrenoreceptor pathway and more specifically the monoamines and their metabolism in the brain (Shelton, 2007). However 50% of individuals do not respond to the current antidepressants (Berton and Nestler, 2006). Moreover, overweight and obese individuals do not respond to current antidepressant treatments suggesting other molecular pathway being involved in the development of depression for this subpopulation (Papakostas et al., 2005). As an aside we wanted to determine whether we could identify novel targets for the development of antidepressant treatment for depression that is caused by dietary factors.

Given the increase RNA expression of GPR40 (Fig. 5.19), I wanted to ascertain whether GPR40 is located upstream of the cAMP/PKA/PDE4A5 signalling pathway that I identified to be involved in the obesity induced depression phenotype. Previously it has been shown that PDEs interact with GPCRs either directly or via the help of scaffolding proteins such as the β-arrestins (Richter et al., 2008). GPR40 has not been shown previously to interact with phosphodiesterase. However, here, for the first time, I show that GPR40 interacts with PDE4A5 in vitru in neurons (Fig. 5.24) and in vivo in the hypothalamus (Fig. 5.28). I then
demonstrated that PDE4A5 translocation at the membrane fraction was time dependent subsequent to palmitic acid treatment of neuronal cells (Fig. 5.30). However, it was not affected by oleic acid treatment (Fig. 5.31), suggesting a differential effect of the fatty acids of different chain length and degree of saturation. In line with these data is the concept that PDEs contribute to the compartmentalization of signalling, many PDEs show unique subcellular distributions due to their ability to form complexes with different receptors (Conti et al., 2003; Houlsay and Adams, 2003). Given the discovery of a free fatty acid receptor in the hypothalamus being involved in the regulation of mood disorders, the inhibition of this receptor and whether this could protect from the dietary obesity induced depression phenotype was investigated next. In vivo i.c.v. administration of GPR40 antagonist in the hypothalamus of mice that were on HFD tended to partially reverse the HFD induced depression phenotype usually observed (Fig. 5.39). However, the difference was not statistical significant and more experiments should be performed with larger cohorts of animals per group.

Despite the potential role of GPR40 signalling in the hypothalamus for lipid sensing that controls energy balance and food intake (Lam et al., 2005b; Obici et al., 2002; Pocai et al., 2006) in this study it is shown for the first time that it might also play an important role in mood disorders such as depression. The data presented in this study set GPR40 as a potential clinical candidate that play a major role in the neurobiology of depression due to diet. This result set the stage for the concept that centrally located mechanisms of fatty acid receptors in the brain can promote signalling that is related to mood disorders as opposed to the traditional view that they regulate physiological pathways in the periphery, such as insulin secretion (Itoh et al., 2003). Hence, in light of this, the development of an inhibitor for the GPR40 that could be used as an antidepressant should be considered. Drugs that selectively block the activation of the free fatty acid signalling in the brain could treat depression even faster than the usual antidepressant drugs and such drugs would be very beneficial for obese and overweight individuals that exhibit resistance to different antidepressant treatments. The identification of the GPR40 as a potential target and the blockage of this target that can rescue the dietary obesity induced
depression may also have repositioning potential for the treatment of depressive disorders associated with pathophysiological changes in cAMP signalling pathway.

What is the role and importance of the interaction between GPR40 and PDE4A5 could be answered with the development of specific inhibitor that disrupts this interaction. Small-molecule inhibitor that selectively target the interaction of the PDEs with free fatty acid receptors could represent a new generation of antidepressants with greater specificity for the overweight or obese individuals or temporally protracted and severe forms of depression due to obesity.

Even though in our study we focused on the role of GPR40 as it showed the most robust RNA increase, other free fatty acid receptors showed a similar increase and it still remains to be determined whether other free fatty acids have a similar effect on the dietary obesity induced depression phenotype. Further studies examining the underlying mechanisms of how activation of different free fatty acid receptors in the brain can activate pathways that can lead to depression should be characterized.

6.13. A potential model for the differential effect of fatty acids on the depression phenotype via GPR40 activation

Various underlying pathophysiological mechanisms may explain our findings, and studies with a mechanistic focus are urgently needed to attain a better understanding of the pathophysiological link between obesity and depression. The adrenergic receptor signalling has long served as prototypes for understanding the function and regulation of GPCRs (Rockman et al., 2002). Many reports indicate that activation of the same GPCR can have distinct and often opposing effects. The specificity of the ligand binding for the signal compartmentalization, coupling and activation may account for the divergent biological effects. Ligand binding on GPCR leads to its activation and interaction with G proteins intracellularly. Different ligand binding induces the assembly of distinct macromolecular signalling complexes with transducer scaffold proteins on the receptor. Moreover, different ligands promote differential conformation
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Final Discussion

of B-arrestins that leads to distinct functional outcomes (Zimmerman et al., 2012).

Furthermore, the function of GPCRs is highly regulated by their agonist stimulated phosphorylation by both second messenger stimulated kinase PKA or PKC and the specialized G protein coupled kinases GRKS (Rockman et al., 2002). PKA phosphorylation of GPCRs which is activated by ensuing rise in cAMP levels has been shown to switch the coupling of the receptor from Gs to Gi (Daaka et al., 1997; Lefkowitz et al., 2002; Zamah et al., 2002; Baillie et al., 2003). Phosphorylation of GPCRs by GRKs promotes binding of arrestins to the phosphorylated receptors. β-arrestin desensitizes the receptor by sterically interdicting signalling to the G protein as well as serves an adaptor that links the receptor to a variety of signalling pathways (Freedman and Lefkowitz, 1996; Goodman et al., 1998; Miller and Lefkowitz, 2001; Krupnick and Benovic, 1998). The recruitment of the β-arrestin scaffold protein bound with PDE4 at the activated GPCRs regulates the PKA membrane activity that, in turn, regulates the switching from Gs to Gi (Baillie et al., 2003; Zamah et al., 2002). Moreover, the recruitment of β-arrestin bound to PDE4 target the degradation of the cAMP at the membrane fraction (Bolger et al., 2003a; Perry et al., 2002) as well as inhibiting further cAMP production by inhibiting the coupling of the receptor with Gs (Perry et al., 2002; Houslay, 2001; Houslay et al., 1998; Houslay and Adams, 2003).

A similar hypothalamic mechanism may underly the development of depression following the consumption of a diet high in saturated fatty acids. GPR40 has been shown to activate different pathways by the binding of different ligands since unsaturated fatty acids stimulate, whereas saturated fatty acids inhibit, cell proliferation via GPR40 in a cultured human breast cancer cell line (Hardy et al., 2000) (Hardy et al., 2003) (Hardy et al., 2005). The lack of ligand discrimination of this receptor supports this hypothesis. The data presented in the study further support that as it was shown that different fatty acids have a different end result on the PKA activity. GPR40 has been shown to bind both palmitic and oleic acid (Briscoe et al., 2003). Ligand binding on the GPR40 might have a differential effect on the macromolecular complexes intracellularly on
the receptor with palmitic acid leading to the coupling with Gi protein and oleic acid with Gs.

Abundance of palmitic acid in the hypothalamus after the consumption of a HFD might leads to quantitatively more binding of palmitic acid on GPR40. This might result to the intracellular macromolecular complex on GPR40 with Gi that inhibits cAMP generation. Moreover, GRK2 phosphorylation of GPR40 might signal the translocation of β-arrestin bound to PDE4A5. This effect will further lead to the switch to Gi signalling and simultaneously the PDE4A5 further degrades and downregulates the cAMP at the membrane. Therefore, the GRK/β-arrestin/PDE4A5 system is not restricted to dampening the rate of cAMP generation but it also increases the local rates of cAMP degradation and this is accomplished by the β-arrestin mediated recruitment of a phosphodiesterase to the receptor that further regulates PKA activity (Perry et al., 2002). The PDE4A5 is therefore playing a pivotal role in underpinning the compartmentalization of the cAMP signalling. This hypothesis is in agreement with our data where HFD leads to increase PDE4 activity at the membrane fraction. The reduction of the cAMP signalling leads to the downregulation of the PKA/CREB pathway that I also observed in the model. On the contrary under normal conditions, consumption of a diet rich in PUFAs might lead to the binding of oleic acid on GPR40 that leads to the coupling of the receptor with Gs that results in the production of cAMP. This model suggests a mechanism where some fatty acids have an antidepressant effect whereas other a depressant effect.

Another possible explanation would be the existence of another receptor that interacts with GPR40 and its one is coupled with either Gi or Gs. Although considerable biochemical and biophysical data are consistent with monomeric GPCRs binding and activating G proteins (Ernst et al., 2007; Whorton et al., 2007) several recent studies suggest that G protein coupling in cell membranes involve the formation of homomeric and heteromeric GPCR complexes (Han et al., 2009; Lopez-Gimenez et al., 2007; Carriba et al., 2008; Vilardaga et al., 2008). Receptor dimerization is one of the most common themes in biology therefore we cannot exclude the interaction of GPR40 with another receptor and this imbalance might result to the development of depression. A possible
candidate might the GPR20 which is an orphan GPCR that is expressed in the brain and in various types of neuroblastoma and is constitutively active and activate Gi proteins leading to the downregulation of the cAMP signalling (Hase et al., 2008). Another possible candidate is GPR21 that has been shown to be highly expressed in the hypothalamus and play a central role in the regulation of energy balance as the GPR21 knock out mice are resistant to obesity induced depression and insulin resistance (Osborn et al., 2012). In support to this hypothesis it has been shown that serotonergic and glutamatergic receptor complexes regulate the balance between Gi and Gq signalling that when the balance between these two receptors is disrupted it can lead to psychosis (Fribourg et al., 2011). A similar phenomenon might happen in the hypothalamus with GPR120 that mainly binds unsaturated fatty acids and GPR40 that binds mainly saturated fatty acids. Both receptors and their downstream scaffold protein β-arrestin2 were shown to be involved in inflammasome inhibition induced by ω-3 FAs (Yan et al., 2013). A imbalance in the signalling cascade coming from the GPR40 and GPR120 receptor under HFD consumption might be responsible for the aberrant signalling that might lead to the development of depression. Further investigation whether GPR40 interacts with another GPCR to regulate depression should be studied. Figure 6.1 represents a schematic of the proposed model that the different fatty acids can lead to the development of depression.
Figure 6.2. Potential model for the dietary obesity induced depression phenotype. A schematic representation of the potential model via which the dietary fatty acids such as unsaturated (USFAs) or saturated (SFAs) bind to GPR40 and differentially regulate the PKA activity that results in gene expression that might affect mood disorders. Picture at the top was taken by [http://www.yourhormones.info/glands/hypothalamus.aspx](http://www.yourhormones.info/glands/hypothalamus.aspx)
6.14. The evolutionary relationship of diet with mood disorders

Obesity reached epidemic proportions worldwide, with incidence rates above 20% in most of the western countries representing a major public health burden (Swinburn et al., 2011). The rise in obesity rates is fuelled by the mismatch between an inherited genetic predisposition for survival in environments where food supply is limited and the current obesogenic environment where reduced physical activity and excess energy intake caused by western diet consumption prevails (Prentice and Jebb, 1995). Modern food processing has resulted in the mass production of cheap, energy-dense foods that are generally high in refined sugars and fats but low in fibre and essential nutrients (Prentice and Jebb, 2003). Putting the psychiatric disorders in the context of evolution I would like to mention “With regards to the intake of essential fatty acids, the modern diets of post industrialized societies appears to be discordant with our genetic pattern and may contribute to the increased prevalence rates of major depression” “Because changes in the dietary intake of essential fatty acids appear to be able to directly influence central nervous system function, these nutrition factors should be further investigated in connection with psychiatric disorders” (Perica and Delas, 2011).

In this context, our study suggests that in the past the free fatty acid receptors in the hypothalamus might have been used for the regulation of appetite via the direct binding of different fatty acids. However over time and the development of a complex hormonal system that tightly regulates the energy balance the free fatty acid receptor pathways in the brain were degenerate to serve for other purposes in our case the development of mood disorders such as depression.
6.15. Final Conclusions

The findings in this study revealed for the first time that obesity, either dietary or genetic is a causative factor for the development of depression and identified a novel molecular mechanism that this phenomenon occurs in mice. This effect is independent of inflammatory pathways and any increase in the body weight and it occurs at a very early stage of obesity. At the mechanistic level high fat diet down-regulates the PKA signalling in the hypothalamus of WT mice by increasing cAMP PDE activity the sole family of enzymes that degrade the cAMP. HFD up-regulates the RNA, protein and phosphorylation state specifically for the PDE4A5 isoform in the hypothalamus. The loss of PDE4A gene in vivo rescues the dietary and genetic obesity induced depression phenotype. The increase PDE4 activity observed at the membrane fraction of hypothalamic samples fed HFD was abolished in PDE4A−/− mice suggesting the important role of the PDE4A gene and the lack of compensatory mechanisms from the other isoforms. Therefore, the biochemical impairment of the PDE4 activity had in vivo functional consequences reflected by the different behavioural paradigms. By exploring the role of PDE4A5 in the hypothalamus we were able to identify its importance in appetite disorders as overexpression of PDE4A5 with the consequence of the downregulation of the cAMP levels in vivo led to severe obesity and hyperphagia.

I also present a novel finding where diet rich in palmitic acid was able to partially induce a depression phenotype whereas a diet rich in oleic acid did not. By exploring the role of the palmitic acid in depression we were able to see the direct effect of palmitic acid in depression after single stereotactic injections in the hypothalamus. The present study provides evidence for the first time of the role of a free fatty acid receptor (GPR40) regulating mood disorders in mice. The interaction of GPR40-PDE4A5 in the hypothalamus implies the important role of this interaction in depression behaviours. GPR40 RNA expression was upregulated in the hypothalamus of mice that consumed HFD. Strikingly, GPR40 antagonist administration specifically in the hypothalamus was able to partially block the development of depression phenotype observed after the consumption of a HFD.

Taken together, these data demonstrate that both PDE4A5 and GPR40 may provide potential targets for the development of antidepressant treatment
specific for the obese or overweight population or population that consume western diet. The data in this study suggest that GPR40 may form a mechanistic link between diet, obesity, and depression. An attractive element of the dietary influence for depression is that it is relatively easy to change this factor compared to gene changes or reducing life stress that are also causative factors for the development of depression. This study provides new and important perspectives regarding underlying mechanisms that may relate to linkages between western dietary habits and mood disorders. In industrialized countries, it is recommended to change diets rich in saturated fat as well as increase exercise to prevent depression and there is a considerable interest in understanding the molecular pathways of how food participates to mental disorders and especially depression and the molecular mechanism presented in this study might be one explanation. The obesity epidemic illustrates how food, particularly low-cost food high in saturated and trans fat can shape our bodies and moreover affect our general mental health status.

In conclusion, even though neuronal and behavioural responses to nutrients are complex and unclear here we provide novel insight into a mechanism through which a specific fatty acid is able to partially mediate the development of depression phenotype. This occurs via the down-regulation of the cAMP/PKA signalling cascade and the activation of a PDE4A5 and a free fatty acid receptor in the brain (GPR40). The PDE4A5-GPR40 interaction in this model of depression offers a novel pharmacological approach in the treatment of depression as targeting a free fatty acid receptor for the treatment of depression has never been investigated. Moreover, evidence in this study implicates that targeting the PDE4A5 in the hypothalamus might be a therapeutic target for eating disorders. How diet influences mood disorders is unknown but this study set the important role of phosphodiesterases and fatty acid receptors to link the gap.
6.16. Future directions

6.16.1. The role of specific diets in memory impairment

More studies directed towards the mechanistic basis of these findings should be performed. It is suggested that mood disorders are associated with a distinct pattern of cognitive impairment, including memory deficits (Arendash et al., 2001). Rolipram has been shown to facilitate the establishment of long-term potentiation which leads to improved learning and memory and act as a cognitive enhancer (Barad et al., 1998; Rose, Hopper et al. 2005; Rutten, Prickaerts et al. 2008). The important role of PDE4A isoforms in synaptic plasticity, memory processing and cognition has been described (Ye, Jackson et al. 2000; Ye, Conti et al. 1997; Takahashi, Terwilliger et al. 1999). HFD has been shown to impair learning and memory in rats (Winocur and Greenwood, 2005; Pathan et al., 2008). Whether diet rich in specific fatty acids might have a similar effect on memory should be further investigated. Furthermore investigation in our model of depression and whether memory impairment is needed and whether it is rescued after the loss of PDE4A gene in vivo.

6.16.2. The role of exercise in depression

Another interesting follow up of this study would be the role of exercise in depression phenotype after HFD. Several experimental studies have documented the preventive effect of exercise in animal model before the installation of obesity/metabolic syndrome (Arvola et al., 1999; Frisbee et al., 2006). Exercise is also able to reverse the metabolic syndrome that occurs in rats that were fed a HFD (Touati et al., 2011). Voluntary wheel running increased cell proliferation and survival in the hippocampal dentate gyrus in mice (van Praag et al., 1999) as well as cell proliferation in rats (Trejo et al., 2001) suggesting an antidepressant effect. Little exercise has been shown to have a huge benefit reversing the aging and infection-induced memory deficits (Barrientos et al., 2011). It has already been shown that postnatal treadmill exercise rescues the depression phenotype of maternal-separated rat pups via the decrease of the apoptotic neuronal death and cell proliferation (Baek et al., 2012). That rescue was accompanied with an increase of serotonin synthesis and tryptophan
hydroxylase, both molecules playing very important role in depression (Baek et al., 2012). Whether exercise is adequate to reverse the HFD induced depression phenotype should be further studied.

### 6.16.3. The role of microbiota in the development of depression

“The concept that the gut and the brain are closely connected, and that this interaction plays an important part not only in gastrointestinal function but also in certain feeling states and in intuitive decision making, is deeply rooted in our language” (Mayer, 2011). From the revolutionary data that revealed that 9 out of the 10 cells in our human body are not human the emerging role of microbiota in different physiological pathways is arising. Gut, the home of most of them has a wide variety of different fatty acid receptors that can be stimulated and induce different molecular pathways. In addition to the obvious role of the gut in the digestion and absorption of nutrients it has an important sensing and signalling role in the regulation of energy homeostasis (Badman and Flier, 2005) which occurs via neuronal and endocrine pathways.

The role of gut microbiota has been implicated in many disorders and spectrums as its composition relates to human disease (Clemente et al., 2012; Kau et al., 2011; Round and Mazmanian, 2009). The diversity of the microbiota can be considered in terms of both richness (the number of species per sample) and evenness (the relative abundance of species). It has been shown that individuals with low bacterial richness are characterized by increased adiposity, insulin resistance and dyslipidemia and a more pronounced inflammatory phenotype in comparison to individuals with higher bacterial richness (Le Chatelier et al., 2013). Microbiota in the gut can secrete different fatty acids that are inserted in the plasma and via the circulation can enter different organs to regulate different molecular pathways. Different diets have been shown to shape gut bacteria ecology and diversity (De Filippo et al., 2010; Le Chatelier et al., 2013; Ou et al., 2013b). HFD consumption has been linked with reversible alterations in the mouse distal gut microbiome (Turnbaugh et al., 2008). Different microbiome due to different diets might result in the expression of different fatty acids from the microbiota that can cross the BBB and might present...
another pathway for the development of mood disorders that should be further investigated. Whether overweight and obese populations have different microbiota than the normal weight population and whether this difference might play a role for the development of depression should be further investigated. It has already been shown that the manipulation of specific bacteria in the gut can work as an anti-obesity drug. Manipulation of gut bacteria that express specific beneficial fatty acids and metabolize in a higher rate the saturated might represent a new therapeutic intervention to treat depression and mood disorders. Therefore the molecular mechanism that link diet, gut, microbiota, metabolites and mood disorders should be further investigated and whether it plays a role in this model of depression.
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