Cross-talk between insulin and serotonin signaling in the brain : Involvement of the PI3K/Akt pathway and behavioral consequences in models of insulin resistance

Ioannis Papazoglou

UNIVERSITÉ PARIS-SUD

ÉCOLE DOCTORALE : "Signalisations et Réseaux intégratifs en Biologie"

Laboratoire de Neuroendocrinologie Moléculaire de la Prise Alimentaire

DISCIPLINE : Neuroendocrinologie

THÈSE DE DOCTORAT

Soutenue le 4 juillet 2013

par

Ioannis Papazoglou

"**Cross-talk between insulin and serotonin signaling in the brain:**
Involvement of the PI3K/Akt pathway and behavioral consequences in models of insulin resistance"

"**Dialogue entre les voies de signalisation de l'insuline et de la sérotonine dans le cerveau:**
Implication de la voie PI3K/Akt et conséquences comportementales dans des modèles d'insulino-résistance"

Directeur de Thèse	Prof. Mohammed Taouis	Université Paris-Sud

Composition du jury:

Présidente	Prof. Anne Mantel	Université Paris-Sud
Rapporteurs :	Prof. Serguei Fetissov	Université de Rouen
	Dr. Michèle Guerre-Millo	Centre de recherche des Cordeliers
Examinateurs :	Dr. Xavier Fioramonti	Université de Bourgogne
	Dr. Ralf Jockers	Institut Cochin
	Dr. Raymond Mongeau	Université Pierre et Marie Curie
	Dr. Claire-Marie Vacher	Université Paris-Sud

Remerciements

Je tiens tout d'abord à exprimer toute ma gratitude au Prof. Mohammed Taouis, Directeur du Laboratoire de « Neuroendocrinologie Moléculaire de la Prise Alimentaire », pour avoir accepté de diriger cette thèse. Je lui suis particulièrement reconnaissant de m'avoir guidé, soutenu et, au besoin, remotivé tout au long de ce travail doctoral.

Ma reconnaissance va également au Dr. Gerozissis pour m'avoir accepté en thèse.

Je remercie le Dr. Claire-Marie Vacher, sans qui la réalisation de la dernière partie du programme de cette thèse et son aboutissement n'auraient sans doute pas été possibles. J'ai pu apprécier sa disponibilité, sa pédagogie et sa gentillesse. C'est grâce à elle que je suis devenu le scientifique que je suis aujourd'hui.

Je remercie le Prof. Anne Mantel pour avoir accepté d'être Présidente de mon jury de thèse.

Je tiens à remercier le Prof. Serguei Fetissov et le Dr. Michèle Guerre-Millo pour m'avoir fait l'honneur et le plaisir d'évaluer et de critiquer ce travail malgré les courts délais que nous avons sollicités.

J'adresse également tous mes remerciements aux examinateurs de cette thèse, les Drs. Xavier Fioramonti, Ralf Jockers et Raymond Mongeau pour avoir accepté de participer au jury de cette thèse et pour l'intérêt qu'ils ont porté à ces travaux.

Je remercie enfin tous les membres, présents et passés, du laboratoire de « Neuroendocrinologie Moléculaire de la Prise Alimentaire » et du « Vekrellis Lab, Basic Neurosciences» pour leur amitié et collaboration pendant cette thèse.

Publications

Xu J., Xilouri M., Bruban J., Shioi J., Shao Z., <u>Papazoglou I.,</u> Vekrellis K., Robakis N.K. (2011). Extracellular progranulin protects cortical neurons from toxic insults by activating survival signaling. Neurobiol Aging. 32(12):2326.e5-16. (see Annex 1)

<u>Papazoglou I.,</u> Berthou F., Vicaire N., Rouch C., Markaki E.M., Bailbe D., Portha B., Taouis M., Gerozissis K. (2012). Alteration of hypothalamic insulin signaling in a type 2 diabetes model associated with a defect in the serotonergic system. Mol Cell Endocrinol. 350(1):136-44.

<u>Papazoglou I.,</u> Jean A., Aubourg A., Gertler A., Taouis M., Vacher C.M. High fat diet induces a reversible depressive like behavior in rats, associated with a down-regulation of the PI3K/Akt/GSK3β pathway in the dentate gyrus. (in preparation)

Communications

<u>Papazoglou I.,</u> Vicaire N., Berthou F., Gerozissis K. and Taouis M. (2010) Molecular mechanisms involved in brain Insulin signaling associated with metabolic and related dysfunctions. Journée de l'école doctorale "Signalisations et Réseaux intégratifs en Biologie", Faculté de Médecine Paris Sud, 28 May 2010, Paris, France

Gerozissis K., Berthou F., Rouch C., Vicaire N., Papazoglou I., Bailbe D., Portha B., Taouis M. (2010) Reduced hypothalamic insulin receptor expression and insulin-dependent Akt phosphorylation in a type 2 diabetes model associated with a defect in the serotonergic system. 46th EASD Meeting, 20-24 September 2010, Stockholm, Sweden.

Papazoglou I., Vicaire N., Berthou F., Gerozissis K. Taouis M. (2010) Molecular mechanisms involved in brain Insulin signaling associated with metabolic and related dysfunctions. "Neuroscience Days" Research Meeting of the Hellenic Society for Neuroscience, 1-2 October 2010, Athens, Greece.

Papazoglou I., Vicaire N., Gerozissis K. Taouis M. (2012) Serotonin-Insulin signaling cross-talk in a human neuronal cell line. 8th FENS Forum of Neuroscience, 14-18 July 2012, Barcelona, Spain.

Table of contents

List of Abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-Hydroxytryptamine (=serotonin)
8-OH-DPAT	8-hydroxy-N,N-dipropyl-2-aminotetralin
Aa	Amino-acid
AC	Adenylate cyclase
Agrp	Agouti-related protein
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AMPK	5'-AMP-activated protein kinase
aPKC	Atypical protein kinase C
ARC	Arcuate nucleus
ATM	Ataxia telangiectasia mutant
BAD	Bcl-2-associated death promoter
BBB	Blood Brain Barrier
BMI	Body Mass Index
C3G	Guanine nucleotide exchange factor
CA1-3	Ammon's Horn (*Cornu Ammonis*) 1-3
cAMP.	Cyclic Adenosine monophosphate
Casp9	Caspase 9
Cbl	Ecotropic retroviral transforming sequence homologue
cDNA	complementary DNA
CHR2	Cytokine homology regions 2
CNS	Central nervous system
Crk	CT10 regulator of kinase
cSH2	C-terminal SH2
DAG	Diacylglycerol
DG	Dentate gyrus
DNA-PK	DNA-dependent protein kinase
DR	Dorsal raphe
eIF	Translation initiation factor

EMT	Extra-neuronal monoamine transporter
eNOS	Endothelial Nitric Oxide Synthase
ER	Endoplasmic reticulum
ERK (MEK) 1/2	Extracellular-signal-regulated kinases 1/2
FNIII	Membrane-proximal fibronectin type III
FOXO1	Forkhead box protein O1
FST	Forced swim test
FYVE	Fab1p, YOTB, Vac1p, EEA1 (early endosomal antigen 1)
GABA	γ-Aminobutyric acid
GIRK	G-protein-coupled inwardly rectifying K+ channels
GLUT1-4	Glucose transporter 1-4
GPCR	G-coupled protein receptor
Grb2	Growth-factor-receptor-bound protein-2
GS	Glycogen synthase
GSK3α/β	Glycogen synthase kinase α/β
HGP	Hepatic glucose production
IDE	Insulin-degrading enzyme
IgD	Immunoglobulin-like domain
IGF-IR	Insulin-like growth factor-I receptor
IKK	Inhibitor of nuclear factor kappa-B kinase
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit β
INPP	Inositol polyphosphate phosphatase
IR	Insulin Receptor
IRS	Insulin Receptor Substrate
ISH	*In situ* hybridization
JAK2	Janus kinase 2
JM domain	Juxtamembrane domain
JNK	c-Jun N-terminal kinases
L1 domain	Leucine-rich-repeat domain 1
LepRa-e	Leptin Receptor a-e
LHA	Lateral hypothalamic area

MAOA	Monoamine oxidase A
MAPK	Mitogen-activated protein kinases
MC3R and MC4R	Melanocortin receptor types 3 and 4
mCPP	m-chlorophenylpiperazine
Mdm2	Mouse double minute 2 homolog
MN	Median raphe
MTM	Myotubularin
mTOR	Mammalian target of rapamycin
mTORC1/2	Mammalian target of rapamycin complex 1/2
NMDA	N-methyl-D-aspartate
NOS	Nitric oxyde synthase
NPY	Neuropeptide Y
nSH2	N-terminal SH2
OCT1-3	Organic cation transporters
PC12 cells	Pheochromocytoma 12 cells
PDK1	3-phosphoinositide-dependent protein kinase-1
PEPCK	Phosphoenolpyruvate carboxykinase
PH	Pleckstrin Homology
PI3K	Phosphatidylinositol 3-kinase
PIP2 or PtdIns(4,5)P2	Phosphatidylinositol (4,5)-bisphosphate
PIP3 or PtdIns(3,4,5)P3	Phosphatidylinositol (3,4,5)-triphosphate
PKA	Protein Kinase A
PKB	Protein Kinase B
PLC	Phospholipase C
POMC	Proopiomelanocortin
PP2A	Protein phosphatase 2A
PS1	Presenilin 1
PTB domain	Phosphotyrosine-binding domain
PtdIns	Phosphoinositides
PTEN	Phosphatase and tensin homologue
PTP1B	Protein tyrosine phosphatase 1B

PVN	Paraventricular nucleus
PX	Phox homology domain
pY	Phospho- tyrosine
Rab5 and 7	Ras-related protein-5 and 7
RACK1	Receptor of activated protein kinase C 1
RIP neurons	Rat insulin promoter 2-expressing neurons
ROCK1	Rho-associated, coiled-coil containing protein kinase 1
RTK	Receptor tyrosine kinase
S6K1	Ribosomal S6 kinase 1
SERT	Serotonin transporter
SF-1	Steroidogenic factor 1
SFKs	Src family kinases
SGZ	Subgranular zone
SH2 domain	Src Homology 2
Shc	Src-homology-2-containing proteins
SHIP	SH2 domain-containing inositol 5'-phosphatase
SHP2 or PTPN11	Tyrosine-protein phosphatase non-receptor type 11
SNRI	Selective norepinephrine reuptake inhibitor
SOCS3	Suppressor of cytokine signaling-3
SOS	Son-of-sevenless
SPT	Sucrose/Saccharine preference test
SSRI	Selective serotonin reuptake inhibitor
STAT3/5	Signal transducer and activator of transcription 3/5
T2D	Type 2 diabetes
TNFα	Tumor necrosis factor α
TPH2	Tryptophan hydroxylase-2
TrpC channel	Transient receptor potential channels
TSC1/2	Tuberous sclerosis protein 1/2
TST	Tail suspension test
UVRAG	UV radiation resistance-associated gene protein
VDVL	Very-low-density lipoprotein

VMN	Ventromedian hypothalamic nucleus
Vps34	Vacuolar protein sorting 34
WHO	World Health Organization
αCT domain	Carboxy-terminal α-chain domain
α-MSH	α-melanocyte-stimulating hormone

General Hypothesis

▶ The essential roles of insulin and serotonin in the hypothalamic regulation of energy balance have been well established. When insulin resistance occurs, in the case of type 2 diabetes for example, the stimulation of the PI3K/Akt signaling pathway in the hypothalamus is attenuated, and the biological effects of this activation are altered. Serotonin can trigger this signaling cascade in the hippocampus and neuronal cell lines, as it has been shown by several studies. However, it is yet to be investigated if serotonin activates this pathway in the hypothalamus too and if this activation is affected by insulin resistance.

Therefore, we hypothesized that serotonin may activate the PI3K/Akt pathway in the hypothalamus and that a potential cross-talk between insulin and serotonin signaling exists in this brain region.

The methodology used to address this hypothesis and the results obtained are described in details in the first research article included in this thesis.

▶ Cumulative research studies indicate a bidirectional association between the metabolic syndrome, including type 2 diabetes, and depression. Serotonin deficiency and/or impaired action are considered among the main causes of depression. The main target for the treatment of this pathology is the serotonergic system especially in the hippocampus.

Thus, we postulated that insulin and leptin resistance that occur in patients with metabolic syndrome could affect the efficiency of serotonergic function in the hippocampus most possibly via a cross-talk involving the PI3K/Akt/GSK3β signaling cascade.

The methodology used to address this hypothesis and the results obtained are described in details in the second research article which is in preparation and included in this thesis.

INTRODUCTION

CHAPTER 1

Type II diabetes, obesity and depression

1.1. Type 2 Diabetes Mellitus (T2D)

Type 2 diabetes (T2D) (or non-insulin-dependent diabetes) is a major global epidemic that increased with a very high rate these last decades, and is predicted to be exacerbated in the decades to come (Chen et al., 2012a). In 2010, 285 million adults (6,4% of world population) were suffering from diabetes, 90% of them from T2D, and in 2030 they are expected to reach the 439 million adults (7,7% of world population) (Shaw et al., 2010). The prevalence of T2D worldwide is a result of a variety of genetic, epigenetic (Drong et al., 2012), sociological (Stringhini et al., 2012), environmental and behavioral factors (Chen et al., 2012a).

Unlike Type 1 diabetes which is caused by the deficiency of insulin production due to autoimmune degeneration of insulin-producing cells, the pancreatic β-cells, T2D is characterized by insulin resistance (decreased insulin production and efficacy). In T2D patients, insulin is unable to increase glucose metabolism and to inhibit liver glucose production leading to hyperglycemia and subsequent hyperinsulinemia (Kahn, 1994). Thus, insulin plays a crucial role in the regulation of glucose homeostasis through its action in various organs (liver, muscle, adipose tissue, brain, heart) and the impairment of insulin efficiency leads to a wide and complex spectrum of physiological dysregulations as those observed in patients with T2D (Konner and Bruning, 2012; Stumvoll et al., 2005). Consequently, insulin resistance not only impedes energy homeostasis maintenance but also correlates with numerous comorbidities including obesity (Drong et al., 2012), cancer (Fantus, 2011), cardiovascular disease (Ferrannini and Cushman, 2012), neurodegenerative diseases (Craft and Watson, 2004), and depression (Pan et al., 2012).

1.2. Obesity

Obesity is a worldwide epidemic with more than 200 million men and 300 million women over the limit of Body Mass Index [BMI=body weight (kg)/ height^2 (m^2)] for obese. Obesity (BMI>30 kg/m^2) and overweight (BMI between 25 and 30 kg/m^2) are fifth in the list with the major death risk factors globally (WHO, 2008). In addition, people with a BMI in the overweight or obese spectrum exhibit higher risk for other pathologies such as type 2 diabetes (Garber, 2012; Sung et al., 2012), cardiovascular disease (Bogers et al., 2007), cancer (Bray, 2002; Renehan et al., 2006) and musculoskeletal disorders (Wearing et al., 2006). Obesity is a multifactorial disease involving epigenetic and/or genetic factors (Drong et al., 2012; Ramachandrappa and Farooqi, 2011), life style, and, environmental and social factors (Malik et al., 2013; Papas et al., 2007).

Most cases of obesity are characterized by hyperleptinemia and leptin resistance, constituting a vicious cycle resulting in the impairment of leptin-dependent food intake inhibition (and leptin-dependent energy expenditure) leading to exacerbated body weight gain and stronger leptin-resistance (Konner and Bruning, 2012; Myers et al., 2010).

1.3. Depression (or Major Depressive Syndrome, MDS)

Over 350 million people worldwide are suffering from depression, which is the leading cause of disability (WHO, 2012). Depression is a complex pathology and very difficult to characterize due to its highly variable course, inconsistent patient response to treatment and no clear established mechanism (Belmaker and Agam, 2008). The most convincing theory about the establishment of depressive behaviors is the monoamine-deficiency theory. According to this theory insufficient neurotransmission or sensitivity of the monoamines serotonin and noradrenaline can cause depressive syndromes (Belmaker and Agam, 2008). Indeed, pharmacological increase of these monoamines at the synapse by selective reuptake inhibitors (SSRIs/SNRIs) improves depressive like symptoms (Mendels, 1987; Papakostas et al., 2007). In addition, polymorphisms on the gene that encodes serotonin transporter (SERT) (Caspi et al., 2003) and the gene that

encodes the principal enzyme for serotonin biosynthesis, the tryptophan hydroxylase-2, (Zhang et al., 2005) have been associated to depression.

1.4. Type 2 Diabetes, Obesity and Depression

Numerous studies have demonstrated the association between type 2 diabetes and obesity which is attributed primarily to the common signaling pathways of insulin and leptin (Belgardt and Bruning, 2010; Benomar et al., 2005a; Benomar et al., 2005b; Eckel et al., 2011; Kahn et al., 2006; Konner and Bruning, 2012). Moreover, the metabolic syndrome (which includes diabetes and obesity) and depression were shown to bidirectionally correlate in a meta-analysis of epidemiological studies (Pan et al., 2012). Another meta-analysis study gave strong evidence (80% of the studies) that obesity is associated with increased development of depression and a moderate possibility (53% of the studies) of depression being a risk factor of installation of obesity (Faith et al., 2011). Finally, other epidemiological studies reported that patients with T2D have 24% more risk of developing depression in comparison to healthy individuals (Nouwen et al., 2010).

Taken together, these findings suggest a strong link between these pathologies. The cross-talk between insulin and leptin signaling pathways revealed many possible mechanisms that can explain the association between T2D and obesity. However, the mechanisms that underlie the connection between T2D and depression as well as obesity and depression are not clearly understood. Serotonin could be the missing component linking obesity/T2D and depression. Indeed, serotonin, a molecule that plays an important role in depression, has also been found to regulate body weight and glucose homeostasis by targeting similar neural circuits as insulin and leptin (Williams et al., 2011). So far, no mechanism has been proposed on how serotonin interacts with the other two hormones to coordinate the overall regulation of energy homeostasis.

CHAPTER 2

Insulin

2.1. General

Insulin is a small peptide hormone (51 amino acids) which regulates glucose metabolism and many other physiological functions. The word "Insulin" derives from the latin word "insula", which means island, and it is the English transfer of the first version in french "insuline".

Insulin was successfully extracted and functionally injected (in dogs) for the first time in 1921 by Banting and Best (Banting et al., 1922). The first injection of a human diabetic patient was performed the following year (Roth et al., 2012). It was the first protein to have its sequence (1951) and tertiary structure (1969) discovered. Since its discovery, it has been a leading molecule for the establishment of many techniques such as radioimmunoassay.

2.2. Production and Secretion

Insulin is synthesized and secreted by the β-pancreatic cells of the islets of Langerhans. When β-cells are stimulated by circulating metabolites (mainly glucose and amino acids), hormones or by the vagus nerve, they secrete insulin into the portal vein and then to the general blood circulation (Goodman, 2009).

Insulin production in the brain has been also demonstrated but it has been a subject of debate and controversy for a long time (van der Heide et al., 2006). A recent article showed that insulin is produced in neurons ending this long lasting debate (Mehran et al., 2012).

2.3. Degradation

Depletion of circulating insulin is an important biological step due to its role in the maintenance of blood glucose levels. Degradation of insulin is catalyzed by a protease,

the insulin-degrading enzyme (IDE). Although IDE is expressed in all insulin sensitive tissues, almost all insulin clearance occurs in liver and kidney (Duckworth et al., 1998).

2.4. Gene, Biosynthesis and structure

Human insulin is encoded by the *INS* gene located at the genetic locus 11p15.5 (Harper et al., 1981) and its sequence was cloned in 1980 by Bell et al. (Bell et al., 1980). In rats there are two insulin genes *ins1* and *ins2* (Cordell et al., 1979).

The product of this gene is preproinsulin, a 110 amino acid polypeptide. (Chan et al., 1976; Steiner et al., 1967). This preliminary molecule undergoes cleavage of the 24 amino acid N-terminal signal-peptide by signal-peptidase enzymes and oxidative folding with the formation of disulfide bonds in the ER. These post-transcriptional modifications result in the more stable and main insulin precursor molecule, proinsulin (Eskridge and Shields, 1983). Finally, proinsulin molecules translocate to the Golgi apparatus where it get packed into secretory vesicles and subjected to maturation. The C-peptide is protease-cleaved by a prohormone convertase and carboxypeptidase E to produce mature insulin.(Docherty et al., 1989; Naggert et al., 1995).
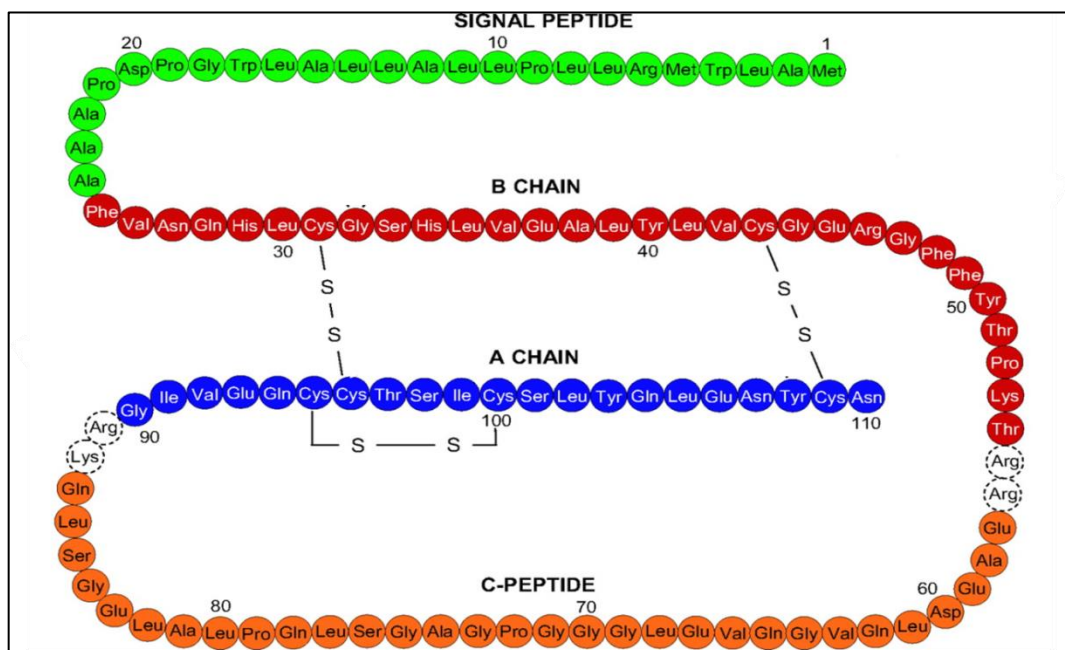
Figure 1: Protein sequence and structure of preproinsulin (Stoy et al., 2007).

Insulin is a globular protein/peptide and one of the smallest functional known proteins. This 51 amino acid peptide hormone has a molecular weight of 5.8 KDa and consists of two chains; A-chain (21 amino acids) and B-chain (30 amino acids). Three disulfide bonds contribute to the formation of the insulin molecule, two between A- and B-chains (A7-B7 and A20-B19) and one intra-A-chain (A6-A11). The crystal structure of this protein has been described as monomer, dimer or hexamer (Hua, 2010).

2.5. Insulin Signaling

2.5.1. Insulin Receptor (IR)

Human insulin receptor cDNA was first cloned by two independent groups in 1985 (Ebina et al., 1985; Ullrich et al., 1985) and the discovery of its sequence and promoter was published few years later (Seino et al., 1989). The insulin receptor gene (*InsR*) encodes 22 exons and 21 introns (Seino et al., 1989). Two different isoforms of insulin receptor, IR_A (predominantly expressed in fetal and cancer tissues) and IR_B, due to alternative splicing of exon 11 have been discovered, with IR_A (which lacks exon 11) having higher affinity and dissociation rate to insulin (Knudsen et al., 2011) .

Upon translation of the *INSR* gene the resulting precursor undergoes glycosylation and cleavage of a 27aa signal peptide in the ER (Ullrich et al., 1985). The occurring proreceptor gets further maturation by the chaperones calexin and calreticulin which includes glycosylation, folding and dimerization. Finally, it translocates to the Golgi apparatus and after proteolysis by furin-protease, a tetraheterotramer is formed consisting of two α and two β subunits (Bass et al., 1998).

Insulin receptor along with insulin-like growth factor-I receptor (IGF-IR) receptor belong to the Class II Receptor Tyrosine Kinase (RTKs) family (van der Geer et al., 1994). IR is a stable heterotetramer formed by two α (~130 kDa) and two β (~95-97 kDa) subunits linked by disulfide bonds (Sweet et al., 1987). The α subunits are extracellular and carry the two insulin binding sites, one on the carboxy-terminal α-chain (αCT) domain and one on the leucine-rich-repeat domain 1 (L1) domain of the receptor, which interact with the α and β chains of insulin respectively (Menting et al., 2013). The β subunits are

transmembrane and carry at their C-terminal an endogenous tyrosine kinase (De Meyts and Whittaker, 2002). In addition, several key tyrosine residues (Tyr[1158]/ Tyr[1162]/ Tyr[1163]) involved in initiating insulin signaling are located in the β subunits (Tavare et al., 1991; Zhang et al., 1991). IR mediates the ligand binding effects via activation of its intracellular related proteins whereas other RTKs directly interact with the intracellular signal transduction molecules (Ullrich and Schlessinger, 1990). One IR αβ-component can also couple with IGF-IR αβ-subunit and form a chimeric receptor where IGF-I binds with higher affinity then insulin (De Meyts and Whittaker, 2002; Soos et al., 1993). IGF-I and IGF-II can bind and activate IR_A/IGF-IR and IR_B/IGF-IR hybrids with high affinity to regulate cell proliferation and migration whereas insulin has very low affinity for both (Benyoucef et al., 2007). Overall, the formation of IR/IGF-IR hybrids favors the action of IGF-I and promotes proliferation- and development. In many types of cancer cells this type of hybrid receptor is the predominant form expressed (Belfiore et al., 2009).
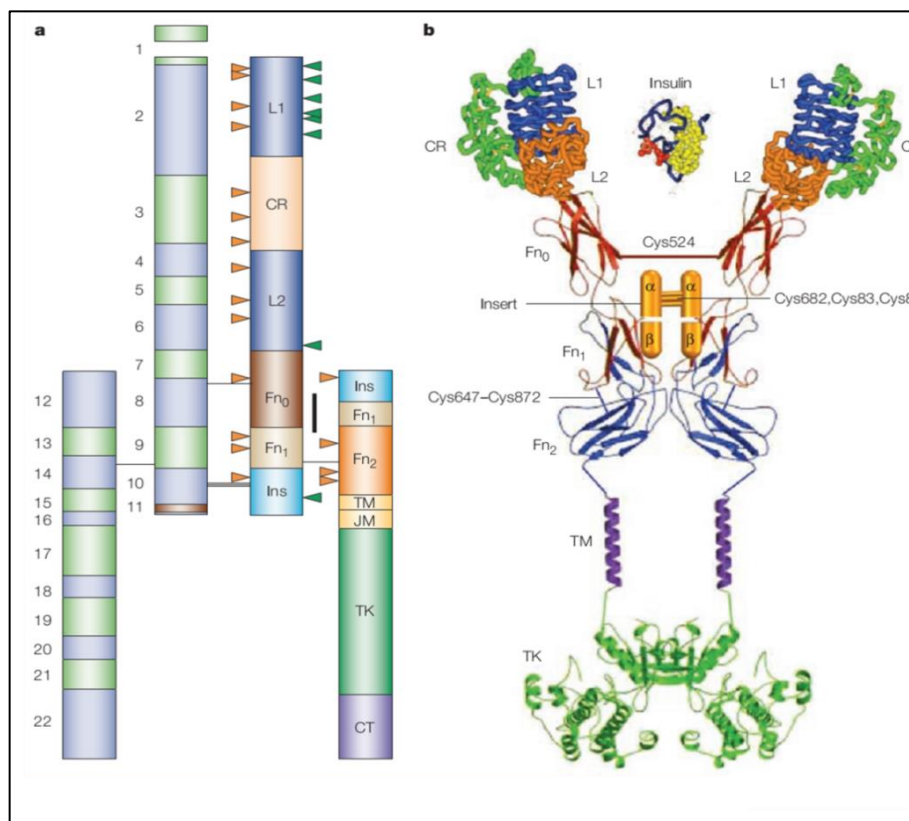
Figure 2:   Insulin Receptor structure (De Meyts and Whittaker, 2002)

a) *left half*: the 22 exons of the insulin-receptor gene, *right half*: protein modules

b) supra-domain organization of the insulin receptor.

Insulin binding to IR induces a conformational change to the receptor which leads to *trans* autophosphorylation of the tyrosine kinase domains (Tyr^{1158}/ Tyr^{1162}/ Tyr^{1163}) (Hubbard, 1997). The phosphorylated tyrosine residues of the insulin receptor serve as docking sites for PH domains and phosphotyrosine-binding domains (PTB domains) (Myers et al., 1995; Wolf et al., 1995). These interactions induce activation of the downstream proteins which in turn trigger respective signaling cascades, such as PI3K/ Akt and Ras/MAPK pathways (Taniguchi et al., 2006).
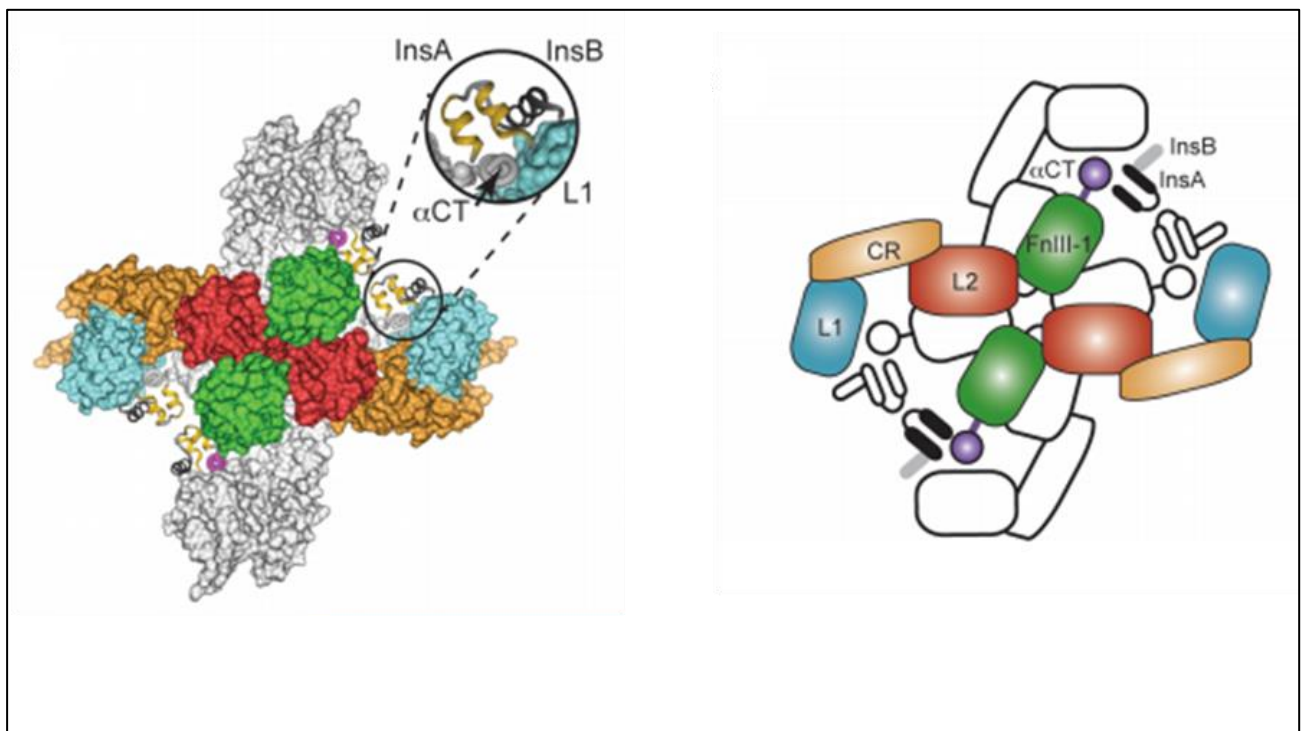
Figure 3: Insulin interaction with Insulin receptor (Menting et al., 2013).

2.5.2. Substrates

a. Insulin Receptor Substrates (IRS)

The IRS protein family contains 6 members (IRS1-6). IRS-1 and -2 are widely expressed in mammalian cells whereas the expression of IRS3, IRS4, IRS5 and IRS6 is limited to specific tissues (Taniguchi et al., 2006). Once the receptor is activated, IRS binds to the phospho-tyrosine residues of the JM domain and membrane phospholipids with the N-terminus PTB and PH domains respectively (Eck et al., 1996; He et al., 1996;

O'Neill et al., 1994). Consequently, phosphorylated tyrosine residues of IRSs serve as docking sites for downstream signaling molecules containing SH2 domains such as: the p85 regulatory subunit of PI3K (Myers et al., 1992) and growth-factor-receptor-bound protein-2 (Grb2) which interacts with son-of-sevenless (SOS) (Myers et al., 1994; Skolnik et al., 1993). Subsequently, these interactions promote the activation of the PI3K/Akt and the Ras/MAPK signaling pathways, respectively (Taniguchi et al., 2006).

b.   Src-homology-2-containing proteins (Shc)

The Shc family contains four members Shc/ShcA, ShcB/Sli, ShcC/Rai/N-Shc, and ShcD/RaLP (Finetti et al., 2009). The three isoforms of ShcA p46, p52 and p66 (Pelicci et al., 1992), after binding to the PTB domain of IR, induce the triggering of Ras/MAPK pathway via Grb2 (Kim et al., 1998) or its inhibition (p66) (Xi et al., 2010). Upon its activation, Grb2 recruits and activates Ras which will subsequently induce the activation of the cascade Raf, MEK, ERK1/2 (Rozakis-Adcock et al., 1992). Shc proteins compete with IRS proteins for the PTB-binding domain of the IR (Gustafson et al., 1995; Wolf et al., 1995).

c.   Other substrates and interacting proteins

Other IR interacting proteins are GRB2-associated-binding protein 2 (GAB), downstream of kinases (DOKs), APS/SH2B, Cas-Br-M (murine) ecotropic retroviral transforming sequence homologue (Cbl) and CT10 regulator of kinase (Crk) (Siddle, 2012). APS and Cbl form a complex which binds to the tyrosine phosphorylated IR via the PH domain of APS. This binding induces tyrosine phosphorylation of Cbl which leads to the recruitment of Crk and the guanine nucleotide exchange factor (GEF) C3G in proximity to the membrane, where C3G activates the TC10 and thus provokes the translocation of glucose transporter 4 (GLUT4) to the plasma membrane (Leto and Saltiel, 2012). Additional molecules that interact with IR but do not undergo tyrosine phosphorylation are JAK2, Grb7/10/14 adaptors, Receptor of activated protein kinase C 1 (RACK1), integrins, β-arrestin and cytohesins (Siddle, 2012).

2.5.3. Negative regulators of Insulin Signaling

In addition to regulation of the PI3K/Akt (Chapter One) insulin signaling can also be modulated at the level of IR and its substrates. IRS serine/threonine phosphorylation by downstream kinases (aPKC, AKT, mTOR, S6K1, ERK1/2, ROCK1, AMPK, GSK3, JNK, IKKβ) feedback regulation induces inhibition of their function (Copps and White, 2012).

a.   Protein tyrosine phosphatase 1B (PTP1B)

PTP1B is a 50 kDa phosphatase which contains a catalytic domain at the N-terminal, a C-terminal ER targeting site and two proline rich domains (PRDs) which are important for protein-protein interactions (Yip et al., 2010). PTP1B is one of the main modulators of the insulin signaling pathway due to the ability to inhibit both IR and IRS (Goldstein et al., 2000; Salmeen et al., 2000). The catalytic domain of PTP1B binds with high affinity to the phosphorylated Tyr^{1162} residue of the activated IR and this interaction results in the dephosphorylation of this pY residue and inactivation of IR (Salmeen et al., 2000). Dephosphorylation of IRS impairs its binding with Crk, GRB2, SHP-2, and the p85 subunit of PI3K (Goldstein et al., 2000). Further, the action of PTP1B reduces the binding affinity of the complex Crk/C3G (Okada et al., 1998) and regulates GLUT4-mediated glucose transport induced by insulin (Chen et al., 1997). $PTP-1B^{-/-}$ and $PTP-1B^{+/-}$ mice have are more insulin-sensitive than wild type littermates and resistant to high fat diet-induced obesity and insulin resistance (Elchebly et al., 1999).

b.   Suppressor of cytokine signaling-3 (SOCS3)

SOCS3 is a member of the SOCS family which includes 8 members (SOCS1-7 and CIS) with similar structure containing a C-terminal SOCS-Box domain and a SH2 domain in the center. The SH2 domain serves as binding site to phosphotyrosine residues (Starr and Hilton, 1998). Insulin signaling activation induces SOCS3 expression (Emanuelli et al., 2000) and activation (Peraldi et al., 2001). Suppressors of cytokine signaling (SOCS), SOCS1 and SOCS3, negatively regulate insulin signaling by blocking IRS tyrosine phosphorylation (Ueki et al., 2004), inducing IRS degradation (Rui et al., 2002) and

inhibiting IR kinase activity (Lebrun and Van Obberghen, 2008). Mice lacking SOCS3 in skeletal muscle are protected against high fat diet-induced hyperinsulinemia and insulin resistance(Jorgensen et al., 2013). Similarly, neuron-specific deletion of SOCS3 prevented the induction of hyperinsulinemia and insulin resistance by high fat diet consumption (Mori et al., 2004).

Figure 4: Insulin signaling map.

2.6. Actions

2.6.1. Periphery

a.   Liver

Liver plays a crucial role in overall glucose homeostasis and the alteration of liver insulin responsiveness (as in insulin-resistant state or type 2 diabetes) leads to increased liver glucose production promoting hyperglycemia. It is well established that insulin plays a key role in liver glucose metabolism by balancing glucose input and output through the inhibition of gluconeogenesis and the activation of glycogen synthesis. Indeed, following meal, glucose is transported via GluT2 (an insulin-independent Glucose transporter) into hepatocytes to be stocked as glycogen or subjected to metabolic modifications to be exported as triglycerides exported into VLDL. Insulin regulates these processes mainly at the gene expression levels. The activation of insulin signaling pathways down-regulates PEPCK expression (key enzyme of gluconeogenesis). Insulin function in the liver mediates blood glucose regulation. Upon glycaemia increase, glucose uptake by the liver follows via the glucose transporter 2 (GLUT2). GLUT2 mediates also glucose release from hepatocytes under hypoglycemic conditions. Insulin acts on the liver via its pathway to induce glucose metabolism. Concurrently, insulin inhibits hepatic glucose production. In addition, activation of the IR/IRS/PI3K/Akt pathway in the liver regulates activity and synthesis of intracellular enzymes. Besides glucose metabolism and glycogen synthesis, this action results to impairment of glycogenolysis, gluconeogenesis, lipolysis, ureogenesis, and ketogenesis and to promotion of lipogenesis and protein synthesis (Goodman, 2009).

b.   Muscle

Insulin action in muscle promotes glucose uptake, phosphorylation and finally storage via transformation into glycogen. Glucose internalization in myocytes is facilitated by insulin induced GLUT4 membrane translocation. In addition, insulin decreases protein degradation by inhibiting protease expression and activity. Moreover, stimulation of the IRS/ PI3K/ Akt pathway augments protein synthesis by increasing

amino acid uptake from the blood via carrier molecules and translation via activation of translation initiation factors 2 and 4 (eIF2, eIF4) (Goodman, 2009).

c. Adipose tissue

Insulin action in adipocytes is multilateral including glucose uptake and transformation to long chain fatty acids or glycogen, esterification, lipolysis and fatty acid uptake from lipoproteins. As in muscle, glucose uptake in adipocytes is mediated by insulin-activated GLUT4 recruitment (Goodman, 2009). In adipocytes, insulin signaling pathway can also transcriptionally and post-transcriptionally regulate the production of leptin, a cytokine produced by this tissue (Lee and Fried, 2009).

2.6.2. Central nervous system

Once thought to be an insulin insensitive organ, the brain has been well demonstrated to be an essential target of insulin. After pancreatic secretion, this hormone reaches the brain by crossing the blood brain barrier and regulates a wide spectrum of cerebral and peripheral functions, the study of which has acquired excessive research attention for many decades now. In addition to glucose metabolism, energy homeostasis and food intake brain insulin action regulates also reproduction, behavior, mood, cognition, neuronal survival, synaptic plasticity, neuronal circuit formation and BBB function. As in hepatocytes, myocytes and adipocytes, insulin binds to IR expressed by neurons and triggers the activation of downstream signaling cascades. Among the multiple cellular functions, glucose uptake and metabolism, protein synthesis and degradation, gene expression and translation are the most essential ones found to be largely regulated by insulin action.

a. Insulin Receptor and signaling in the brain

Insulin receptor is abundantly distributed in the mammal brain. However, the density of the receptor varies among different brain regions (Havrankova et al., 1978; Schulingkamp et al., 2000) and is reduced with ageing (Zhao et al., 2004). IR is more

predominantly expressed in the olfactory bulb, cerebellum, hippocampus, cortex, choroid plexus and the hypothalamus (Marks et al., 1990; Zhao et al., 1999). Both neurons and glial cells express IR (Lowe et al., 1986). Brain insulin receptor exhibits both structural and functional differences with the one found in peripheral organs. The molecular weight and carbohydrate composition of both α and β chains of the IR are found to be lower in the brain in comparison to the periphery (Heidenreich et al., 1983). Glial IR β subunit is bigger than the neuronal one and smaller than the one found in the liver (Lowe et al., 1986). Moreover, unlike peripheral IR, brain IR is not down-regulated when exposed to chronic hyperinsulinemic conditions (Zahniser et al., 1984).

The primary step upon IR activation is the tyrosine phosphorylation of its substrates IRS and Shc. In turn, PI3K/Akt and Ras/ERK signaling pathways are triggered transforming the insulin signal into cell process regulation (Kim and Feldman, 2012).

b. Hypothalamus

Insulin signaling in the hypothalamus is known to modulate hepatic glucose production (Obici et al., 2002b), food intake, fat mass, hepatic insulin sensitivity (Obici et al., 2002a), reproduction (Hill et al., 2010) and behavior (Grillo et al., 2011). Three neuronal subpopulations (POMC, Agrp/NPY, RIP) in the arcuate nucleus (ARC) and one (SF-1) in the ventromedial (VMN) hypothalamic nucleus are found to mediate the metabolic effects of hypothalamic insulin signaling (Grayson et al., 2012; Rother et al., 2012).

i. The Arcuate Nucleus (ARC)

The arcuate nucleus is located at the mediobasal hypothalamus in proximity to the third ventricle and the median eminence, a circumventricular organ (Paxinos and Watson, 2005). The position of ARC is thus optimal for reception and integration of peripheral signals such as circulating hormones by the neurons situated in this area. Inside the ARC two principal neuronal populations that play an important role in the maintenance of energy homeostasis are targeted by insulin, the anorexigenic POMC and the orexigenic Agrp/NPY (Lin et al., 2010). More recently, another distinct subpopulation

of neurons in the ARC, the rat insulin promoter-expressing (RIP) neurons was reported to be targeted by insulin (Rother et al., 2012).

The first population of neurons produces the 31-kD precursor protein proopiomelanocortin (POMC) (Mountjoy, 2010). The main product of this protein in the ARC, among many products, is the α-melanocyte-stimulating hormone (α-MSH), a 13 amino-acid neuropeptide (Mountjoy, 2010). α-MSH acts as an agonist of the G protein coupled receptor (GPCR) melanocortin receptors types 3 and 4 (MC3R and MC4R) (Lu et al., 1994). Stimulation of MC4R by α-MSH in the brain leads to suppression of food intake (Balthasar et al., 2005) and increase of peripheral functions such as energy expenditure, hepatic and muscular insulin sensitivity and insulin production (Fan et al., 2000; Mountjoy, 2010; Rossi et al., 2011). These effects are initiated via POMC projections to other hypothalamic nuclei such as PVN, VMN and the lateral hypothalamic area (LHA) as well as the preganglionic neurons of the spinal cord that express melanocortin receptors (Jacobowitz and O'Donohue, 1978; Mountjoy et al., 1994). Insulin binding to IR expressed by these neurons induces two parallel effects. Firstly, insulin action generates activation of the PI3K (Xu et al., 2005), which results in increased PIP3 levels and subsequent opening of ATP-dependent potassium (K_{ATP}) channels (Plum et al., 2006). Thus, insulin action on POMC neurons induces their hyperpolarization and inhibition of their excitability (Williams et al., 2010). Interestingly, upon insulin-induced activation of Akt, FOXO1 is phosphorylated and translocated from the nucleus to the cytoplasm. This effect prevents the FOXO1-mediated inhibition of POMC gene expression, increasing this way the intracellular levels of this protein and its derivative neuropeptides (Belgardt et al., 2008). However, deletion of IR from POMC neurons has no effect in energy homeostasis (Konner et al., 2007). Re-expression of IR in POMC neurons demonstrated that insulin action in these neurons increases hepatic glucose production (HGP) and energy expenditure (Lin et al., 2010).

The second subgroup, Agrp/NPY, consists of neurons that co-express two anorexigenic peptides: the Agouti-related protein (Agrp) and the Neuropeptide Y (NPY) (Hahn et al., 1998). Agrp is an antagonist of MC3R and MC4R and thus antagonizes the

anorectic action of α-MSH, introducing the opposite effect which is hunger (Lu et al., 1994; Ollmann et al., 1997; Rossi et al., 1998). NPY is a 36-amino-acid neuropeptide (Tatemoto, 1982) which initiates orexigenic action via activation of Y1 (Mullins et al., 2001; Pedrazzini et al., 1998) and Y5 (Cabrele et al., 2000) receptors. Similarly to POMC neurons, insulin acts via its receptor to activate PI3K (Xu et al., 2005). This stimulation leads to hyperpolarization of these neurons via activation of K_{ATP}-channels, in a mechanism analogous to the one in POMC (Konner et al., 2007). FOXO1 promotes the expression of both Agrp and NPY and is blocked by insulin action via activation of the PI3K/Akt pathway (Kim et al., 2006). In addition, insulin signaling activation in this neuronal subgroup is necessary for the suppression of HGP (Konner et al., 2007; Lin et al., 2010).

The RIP-expressing neurons have been recently identified as important regulators of energy homeostasis. They have been found to produce neither NPY nor POMC, but to exhibit orexigenic properties, since their ablation results in hypophagy and reduced body weight (Rother et al., 2012).

i.   The Ventromedial Nucleus (VMN)

The ventromedial nucleus of the hypothalamus (VMN) comprises cluster of neurons which are located in the mediobasal hypothalamus, proximate to the third ventricle and above the ARC (Paxinos and Watson, 2005). In this brain area, there is one specific neuronal group that expresses the nuclear receptor Steroidogenic factor 1 (SF-1), which plays a significant role in the development and function of the VMN (Ikeda et al., 1995; Zhao et al., 2008). Insulin-induced activation of PI3K in SF-1 neurons results in opening of K_{ATP}-channels and inhibition of excitability (Klockener et al., 2011). In addition, IR/PI3K signaling in these cells contributes to impairment of glucose metabolism and increased weight gain (Klockener et al., 2011).
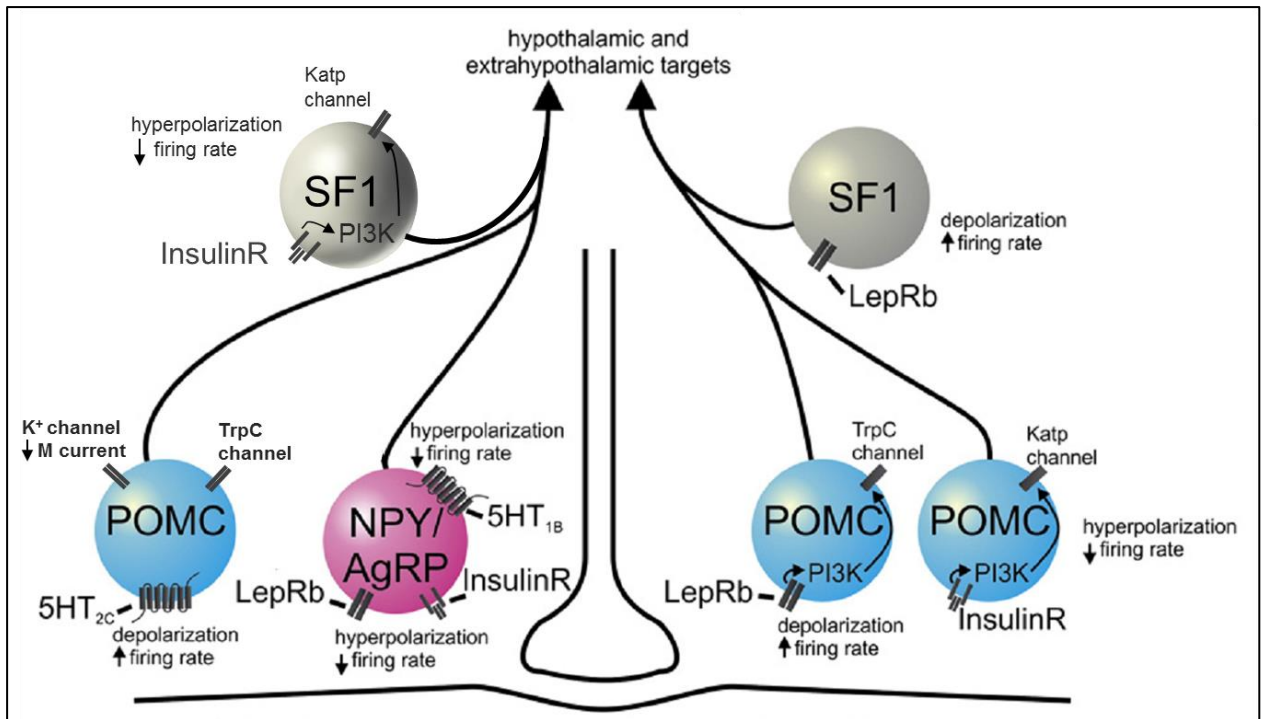
Figure 5: Hypothalamic action of insulin, leptin and serotonin in the VMN SF1 neurons and the Arcuate POMC and NPY/Agrp neurons. Modified from (Williams et al., 2011). Modifications: a) insulin inhibits SF1 neurons firing rate via activation of K_{ATP} channels (Klockener et al., 2011) and b) the depolarization of POMC neurons by 5-HT_{2C} is induced via activation of TrpC channels (Sohn et al., 2011) and K^+channel-mediated M-current inhibition (Roepke et al., 2012).

c.   Hippocampus

Hippocampal regions display a high mRNA expression and protein levels of IR and insulin signaling machinery (Bondy and Cheng, 2004; Folli et al., 1994; Zhao et al., 1999). Insulin action on hippocampal neurons regulates synaptic plasticity (Zhao et al., 2004) by modulating the function of GABAergic (Wan et al., 1997) and glutamatergic receptors (Man et al., 2000; Martin et al., 2012), inhibits pyramidal cell firing (Palovcik et al., 1984) and induces neuroprotective effects (Hui et al., 2005; Sun et al., 2010).

d. Insulin, Blood Brain Barrier (BBB) and glucose uptake

Pancreatic insulin can reach the CNS by crossing the blood-brain barrier with a rate of 0.5 $\mu l*g^{-1}*min^{-1}$. This rate varies depending on numerous conditions such as fasting, obesity and ageing. Insulin transport across the BBB is under the control of a saturable mechanism, which limits the hyperinsulinemic contagion of the brain when blood insulin increases significantly. Less than $1^o/_{oo}$ of intravenously injected insulin is found to enter the mouse brain. A transporter molecule is hypothesized to perform insulin transfer across brain endothelial cells which is yet to be identified (Banks et al., 2012). Insulin uptake varies between different regions. The brain structures with the most elevated BBB crossing are pons-medulla, hypothalamus, hippocampus, striatum, cerebellum, frontal cortex and parietal cortex (Banks and Kastin, 1998).

Insulin effects on BBB include amino acid uptake (tyrosine and tryptophan), hormones uptake (leptin) and protein degradation (amyloid beta 1-42). Glucose uptake in the brain is overall an insulin-independent function. The principal fuel of the CNS reaches the brain by transportation through the BBB. This process is potentiated by GLUT-1 in a process separated from insulin action. Internalization of glucose by brain cells can be facilitated by GLUT-4 after insulin-induced stimulation. However, glucose uptake by neurons and glial cells is primarily a result of insulin-independent procedures via the GLUT-1, GLUT-3 and GLUT-5 (Banks et al., 2012).

2.7. Insulin Resistance

Insulin resistance is generally determined by the loss of sensitivity of targeted tissues to circulating insulin. More precisely, insulin responsive cells' ability to react upon ligand-receptor binding is perturbed and as a result the insulin-induced effects are reduced or impaired. This condition is associated with a number of pathologies including type 2 diabetes mellitus (T2DM), obesity, metabolic syndrome, Alzheimer's disease, Parkinson's disease, depression and cardiovascular disease.

CHAPTER 3

Serotonin (5-HT)

3.1. General

Although its existence and action on smooth muscle contraction was known already during the 19th century, serotonin was isolated for the first time in 1948 by Rapport and colleagues and was given this name due to its source, serum, and its ability to cause vasoconstriction to organs when released (Rapport et al., 1948). Few years later, this molecule was identified to be 5-hydroxytryptamine (5-HT) (Reid and Rand, 1952).

3.2. Brain Serotonin

Brain 5-HT is the subject of excessive research due to its role as a neurotransmitter. It was first discovered in the brainstem in 1964 by Dahlström and Fuxe (Dahlstrom and Fuxe, 1964). Being the most widely distributed neurotransmitter in the brain, it regulates a wide range of functions including behavior, cycle/wake cycle, mood and food intake. As a consequence, impairment of serotonergic production or signaling leads to a large spectrum of pathologies such as anxiety, depression, obesity and schizophrenia.

3.3. Production and secretion

Serotonin amounts in the brain are mainly originated from neuronal clusters located in the midline of the brainstem tegmentum. More precisely, the somata of the 5-HTergic neurons are found in the nine raphe nuclei (B1-9), which are grouped in three major formations: the dorsal raphe (B6, B7), the median raphe (B9, B8, and B5) and the caudal raphe (B1-3). The axons of rostral 5-HTergic neurons project towards the forebrain (ascending projections) and reach numerous regions such as hippocampus, frontal cortex, striatum, hypothalamus and amygdala. Caudal 5-HT producing cells project to the cerebellum and spinal cord (descending projections). Two distinct types of 5-HT axon terminals are found in the rat brain: *fine* axons which have tiny varicosities

(less than 1 μm in diameter) that vary in shape, and *beaded* axons which have large, spherical varicosities (typically 2-3 μm in diameter) connected by thin intervaricose segments (Kosofsky and Molliver, 1987).

Serotonergic neuron activity is feedback regulated by 5-HT-targeted regions via other transmitters including glutamate, acetylcholine, GABA, noradrenaline and neuropeptides (Lesch and Waider, 2012). Along with other amines (including noradrenaline and dopamine), 5-HT is thought to "modulate" rather than "mediate" information exchange. This notably relies on the observations that, in contrast with neurotransmitters such as glutamate and GABA, which transit in neurons exhibiting high variability in firing rates and emitting sharply focused projections, 5-HT is released in a more tonic way (Jacobs and Formal, 1999) in disparate targets.
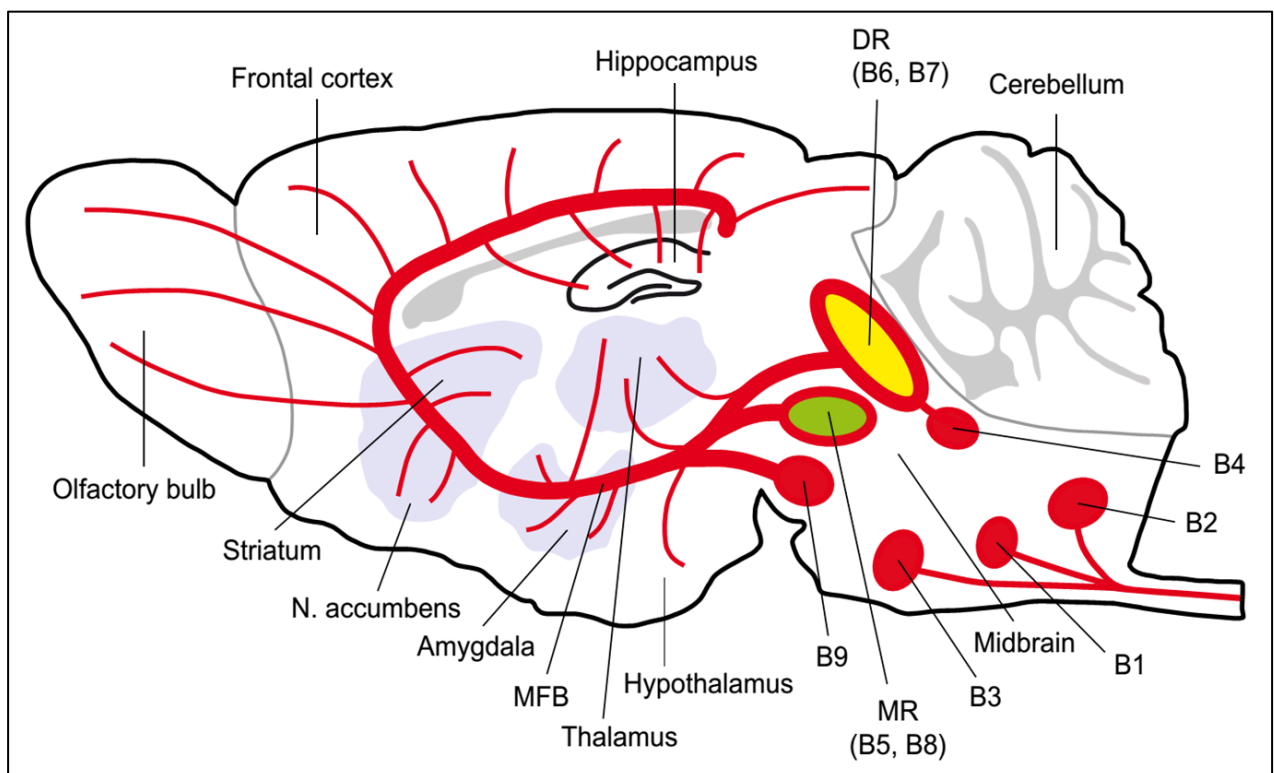
Figure 6: Projections of serotonergic neurons to the spinal cord and periphery (B1-B3) and to forebrain (B4-B9). From Lesch and Waider. (Lesch and Waider, 2012).

Despite its significance, brain 5-HT is only a minority in terms of quantity (~5% of total 5-HT body content). The largest amounts (~90%) of 5-HT in the body of Mammals

are produced, stored and secreted by the enterochromaffin cells of the gastrointestinal mucosa (Gershon and Tack, 2007). Serotonin production is also found in the pineal gland, where it represents the precursor of melatonin during the night, the neuroepithelial bodies of the lungs, the parafollicular cells of the thyroid (Gaspar et al., 2003) and the cells of the intestinal myenteric plexus (Gershon et al., 1965).

3.4. Biosynthesis and structure

Serotonin belongs to the family of monoamines. It is small indolamine (MW 176.2 g/mol) that derives from the enzymatic conversion of the amino acid tryptophan in two steps. The first and rate limiting step, the hydroxylation of tryptophan, is catalyzed by tryptophan hydroxylase (TPH, TPH1 and TPH2, which is more brain-specific) and leads to the production of 5-hydroxytryptophan (5-HTP). Aromatic L-amino acid decarboxylase (AADC) performs the second step, which involves the decarboxylation of 5-HTP. Transcriptional regulation of these enzymes controls the production of 5-HT by serotonergic neurons (Deneris and Wyler, 2012).
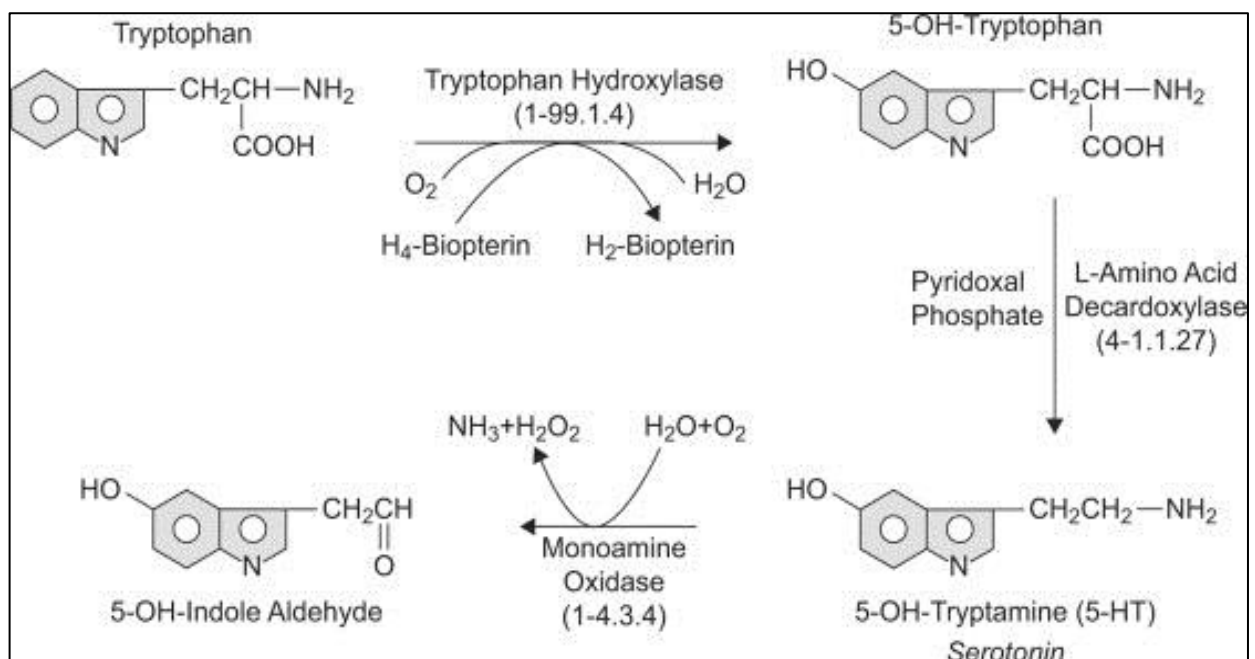
Figure 7: Biochemical pathway of the synthesis of serotonin from tryptophan and its metabolism to 5-OH-Indole Aldehyde (5-HIAA). From E.C. Azmitia (Müller and Jacobs, 2010).

3.5. Degradation and uptake

Membrane-bound mitochondrial flavoprotein monoamine oxidase A (MAO) is the main enzyme that catalyzes the oxidation, and thus the degradation, of 5-HT. This enzyme, via oxidative deamination, converts 5-HT into 5-hydroxyindoleacetic acid (5-HIAA), which is quickly removed from the organism by the urines (Huszti and Borsy, 1968).

Serotonin re-uptake from extracellular fluid into 5-HT terminals mainly occurs via high affinity uptake mediated primarily by the serotonin transporter (5-HTT/SERT) (Blackburn et al., 1967; Blakely et al., 1991; Daws, 2009). Other proteins that exhibit lower affinity but a high capacity for 5-HT uptake, such as the extra-neuronal monoamine transporter (EMT) and the organic cation transporters (OCT1-3), can ensure 5-HT uptake in non-neuronal cells, probably contributing to the 5-HT homeostasis (Baganz et al., 2008; Feng et al., 2005; Gasser et al., 2006; Grundemann et al., 2002; Schmitt et al., 2003).

3.6. Serotonin signaling

3.6.1. Serotonin Receptors

Serotonin receptors are grouped in 7 classes according to their function, structure and intracellular signaling properties. The 6 of them (1, 2, 4, 5, 6, 7) belong to the α-group of rhodopsin of GPCRs, in the amine receptor cluster. The 5-HT3 receptor is an exception since it is a ligand-gated ion channel that belongs to the nicotinic acetylcholine receptor superfamily (Berumen et al., 2012; Fredriksson et al., 2003). The 6 GPCR groups are separated according to the G-protein they couple to. The first group of receptors (5-HT$_{1A-F}$) couples to G$_{i/o}$ proteins, the second (5-HT$_{2A-C}$) couples to G$_q$ proteins, the receptors 5-HT$_4$, 5-HT and 5-HT$_7$ couple to G$_s$ proteins. Finally, it is not yet clear which G-proteins interact with the 5-HT$_5$ receptors (Raymond et al., 2001).

a.   Class 1

Class 1 comprises five members: 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1E}$ and 5-HT$_{1F}$ which couple to G$_{i/o}$ proteins to inhibit the enzyme adenylate cyclase (AC) and decrease the

production of cAMP. 5-HT$_{1C}$ was removed from this group and placed to the second class as 5-HT$_{2C}$.

5-HT$_{1A}$ receptors are distributed ubiquitously in the brain. Presynaptic auto-receptors are detected in the cell bodies and dendrites of serotonergic neurons of the raphe nuclei (Sotelo et al., 1990), which inhibit firing by hyperpolarization via activation of the G-protein-coupled inwardly rectifying K$^+$ channels (GIRK) (Penington et al., 1993; Sprouse and Aghajanian, 1987). Postsynaptic 5-HT$_{1A}$ receptors are located in the forebrain, with higher density in the hippocampus (granule cells of the dentate gyrus) and the septum using a specific ligand, 8-hydroxy-N,N-dipropyl-2-aminotetralin (8-OH-DPAT) (Pazos and Palacios, 1985).

5-HT$_{1B}$ receptor is expressed throughout the brain and is found in higher concentrations in the basal ganglia, striatum and frontal cortex. It serves as axon terminal auto- or heteroreceptors (Hoyer et al., 2002). *In situ* hybridization and Northern blot analysis revealed its expression in other regions such as the septum, the ventral tegmental area, the colliculi, the hypothalamus and the hippocampus (Doucet et al., 1995). 5-HT$_{1B}$ in axon terminals activation inhibits 5-HT and other transmitter release (Riad et al., 2000).

5-HT$_{1D}$, 5-HT$_{1E}$ and 5-HT$_{1F}$ receptors are expressed at very low levels in comparison to 5-HT$_{1A}$ and 5-HT$_{1B}$ and their role is not very well defined due to lack of specific ligands.

b. Class 2

Class 2 comprises 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors, which couple positively to G$_{q/11}$. Due to its structural, functional and signaling transmission properties that resemble more to the ones of 5-HT$_2$ receptor subtypes, 5-HT$_{1C}$ was reclassified in this group. They all couple to G$_{q/11}$ proteins to activate phospholipase C and stimulate the hydrolysis of membrane phospholipids, increasing in parallel the formation of inositol phosphates and diacylglycerol (Bockaert et al., 2006).

5-HT$_{2A}$ receptors are distributed throughout the brain, mainly in the cortex, striatum, and hippocampus. Their location can be on the soma, the dendrites or the axons of pyramidal neurons, interneurons and monoamine releasing neurons (Polter and Li, 2011). 5-HT$_{2A}$ receptors action has been found to oppose to the effects of 5-HT$_{1A}$ in some cases (Li et al., 2004b).

5-HT$_{2B}$ receptor has been detected in the rat CNS in the following regions: cerebellum, lateral septum, hypothalamus and medial amygdala (Duxon et al., 1997). This receptor is also expressed peripherally where it plays an important role in the function of the cardiovascular system (Launay et al., 2002), the liver (Ebrahimkhani et al., 2011) and the intestine (Wouters et al., 2009).

5-HT$_{2C}$ receptors, in rats, are exclusively expressed in the brain, the choroid plexus and the spinal cord (Canton et al., 1996). They are present in the cellular membrane as homodimers (Herrick-Davis et al., 2012) and this dimerization is necessary for their function (Herrick-Davis et al., 2005). In addition, the mRNA of this receptor undergoes editing and results in the production of numerous isoforms with altered affinity to 5-HT and function (Burns et al., 1997).

c.   Class 3

5-HT$_3$ receptors, previously called M-receptor of Gaddum and Picarelli, are Cys loop ligand-gated ion channels, similar to nicotinic acetylcholine (nACh) receptors, and exhibit distinguished structure and functions compared to other six classes (Lummis, 2012). The 5-HT$_3$ receptors are ligand-gated ion channels that trigger rapid neuronal depolarization and excitatory neurotransmission (Derkach et al., 1989) through Ca^{2+} (Hargreaves et al., 1994), Na^+ and K^+ flow (Malone et al., 1991). The two 5-HT$_3$ subunits (A and B) form homo- and hetero-pentameric receptors but only the 5-HT$_{3A}$ subunit is functionally present in the CNS (van Hooft and Yakel, 2003). The use of specific radioligand revealed higher levels of binding in the brainstem, mainly in the solitary tract nucleus. In the forebrain, medium density was found in the olfactory bulb and various subnuclei of the amygdala (Gehlert et al., 1993).

d. Classes 4-7

5-HT$_4$ receptors are mainly expressed in the olfactory tubercle, the caudate putamen, the ventral striatum, the medial habenula and the hippocampus. They are both somatodendritically and axonaly localized (Vilaro et al., 1996). 5-HT$_4$ receptors activate AC and increase cAMP (Dumuis et al., 1988).

The **5-HT$_5$** receptor class includes two receptors, 5-HT$_{5A}$ and 5-HT$_{5B}$ which are detected in higher density in the hippocampus (5-HT$_{5B}$ only in the CA1) (Erlander et al., 1993; Matthes et al., 1993). 5-HT$_{5A}$ is expressed in astrocytes where it inhibits AC (Carson et al., 1996). However the signaling pathways activated by these receptors have not been clearly demonstrated to date (Berumen et al., 2012).

5-HT$_6$ receptors activate AC and are predominantly expressed in the brainstem and to lower extend in forebrain, cerebellum, intestine and heart (Plassat et al., 1993).

5-HT$_7$ receptors induce the production of cAMP by activating AC and are expressed in limbic areas, including hippocampal pyramidal cells (Ruat et al., 1993). It regulates a number of function including mood, thermoregulation, memory and sleep (Hedlund and Sutcliffe, 2004).
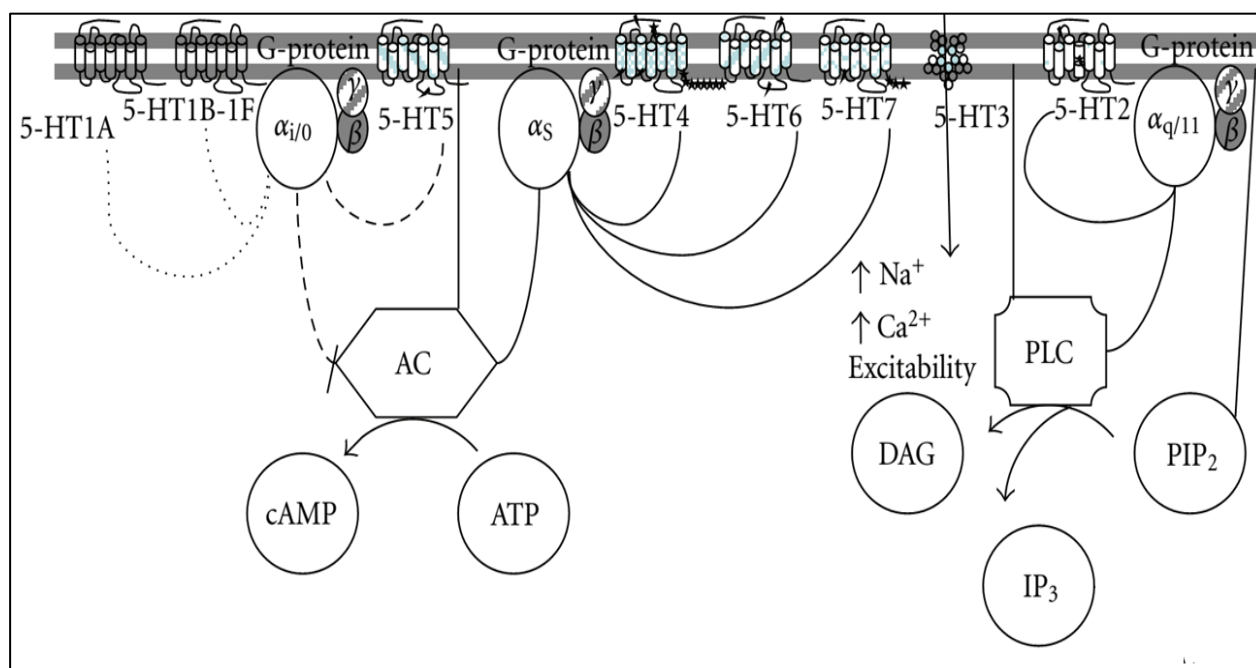

Figure 6: Main signaling pathways induced by the activation of different 5-HT receptors. Modified from Laura Cristina Berumen and colleagues (Berumen et al., 2012).

Receptor (variant)	Population	Principal G proteins	Principal signals	Other G proteins	Other direct signals	Downstream signal (mediator)[b]
5-HT$_{1A}$	Recombinant	G$_{i/o}$ (G$_{i1}$, G$_{i2}$, G$_{i3}$, G$_o$)	AC and PKA (−) gK$^+$ (GIRK) (+) gCa^{2+} (N, P/Q) (−)	G$_z$	PLC, Ca^{2+} and PKC (+) PLA$_2$ and AA (+) AC II (+)	pERK (Ras, PI3K) (+) pAkt (PI3K) (+) JNK (Rho) (+) [87] NHE (Jak2 and CaM) (+) I$_{smooth}$ (+)
	Endogenous (brain)	G$_i$ (DRN) G$_o$ > G$_i$ (Cx, Hip, Hyp)	AC and PKA (Cx, Hip) (−) gK$^+$ (GIRK) (DRN) (+) gCa^{2+} (N, P/Q) (DRN) (−)	G$_z$ (Hyp, Hip)	PLC (Hip) (−) PLA$_2$ (Hip) (+)	pERK (Hip, DRN) (−) pERK (Hyp) (+) pAkt (Hip) (+) gK$^+$ (TWIK-1) (Ecx) [88]
5-HT$_{1B}$	Recombinant	G$_{i/o}$ (G$_{i1}$, G$_{i2}$, G$_{i3}$, G$_o$)	AC and PKA (−)		PLC, Ca^{2+} and PKC (+) nNOS (+) gK$^+$ (Ca^{2+} dependent) (+) gCa^{2+} (voltage dependent) (−)	pERK (Ras, PI3K) (+) pAkt (PI3K and p70 S6 kinase) (+)
	Endogenous (brain)	G$_{i/o}$ (striatum)	AC and PKA (S. nigra) (−)			pERK (+)
5-HT$_{1D}$	Recombinant	G$_{i/o}$ (G$_{i1}$, G$_{i2}$, G$_{i3}$, G$_o$)	AC and PKA (−)		gK$^+$ (Ca^{2+} dependent) (+) gCa^{2+} (voltage dependent) (−) gCa^{2+} (N) (−)	pERK (+)
	Endogenous (brain)	G$_{i/o}$ (Hip, Cx)	AC and PKA (−)			
5-HT$_{2A}$	Recombinant	G$_{q/11}$	PLC, Ca^{2+} and PKC (+) PLA$_2$ and AA (+)	G$_{i/o}$ G$_{12,13}$	cAMP, PKC and CaM (+) PLD (Arf1) (+)	pERK (many pathways) (+) p38 kinase (RhoA) (+) pAkt (PI3K) (+) NHE (Jak2 and CaM) (+) STAT3 (Jak2) (+)
	Endogenous (brain)	G$_{q/11}$ (Cx)	PLC and Ca^{2+} (Cx, striatum) (+) PLA$_2$ and AA (Cx, Hip) (+)	G$_i$ (Cx)	NOS (Cx) (+) gCa^{2+} (L) (Cx) (+) gCa^{2+} (voltage independent) (Cx, astrocytes) (+) gK$^+$ (delayed rectifying) (Cx) (−)	pERK (Src and β-arrestin) (Cx) (+) [89] gNa$^+$ (PKC) (Cx) (−)
5-HT$_{2B}$	Recombinant	G$_{q/11}$	PLC, Ca^{2+} and PKC (+)	G$_{13}$	NOS (+)	pERK (Src and Ras) (+)
	Endogenous (brain)	G$_{q/11}$	PLC, Ca^{2+} and PKC (Cx, astrocytes) (+) PLA$_2$ and AA (+)		NOS (+)	Na$^+$-K$^+$-ATPase (PKC) (−)
5-HT$_{2C}$ (numerous isoforms)	Recombinant	G$_{q/11}$	PLC, Ca^{2+} and PKC (+) PLA$_2$ and AA (+)	G$_{12/13}$ G$_{i1}$, G$_{i3}$, G$_o$	PLD (RhoA) (+) gK$^+$ (GIRK, others) (−) gCl$^-$ (Ca^{2+} gated) (+)	pERK (many pathways) (+) pAkt (PI3K) (+) gK$^+$ (PKC) (−)
	Endogenous (brain)	G$_{q/11}$	PLC, Ca^{2+} and PKC (choroids plexus, Cx) (+)		NOS (choroids plexus) (+) gK$^+$ (delayed rectifying) (choroid plexus, striatum, Hyp) (−) [90]	pERK (Src and β-arrestin) (Cx) (+) [89] gNa$^+$ (PKC) (Cx) (−)
5-HT$_4$ (up to ten splice variants)	Recombinant	G$_s$	AC and PKA (+)	G$_{13}$	RhoA (+) Epac/Rap1 (+) gCa^{2+} (L) (+)	pERK (Src) (+) pERK (PKA and Ras) (+) NHE (Src and Ca^{2+} dependent) (−)
	Endogenous (brain)	G$_s$	AC and PKA (colliculus) (+)	G$_{13}$	RhoA (+) TTX-insensitive gNa$^+$ (Hip, DRN) (+) gCation (globus pallidus) (+) [91]	pERK (Src) (colliculus) (+) gK$^+$ (voltage and Ca^{2+} dependent) (PKA) (Hip, colliculus) (−) I$_H$ current (Hip) (+)
5-HT$_{5a}$	Recombinant	G$_{i/o}$	AC and PKA (−)		PLC, Ca^{2+} and PKC (+) gK$^+$ (GIRK and other K$^+$-channel types) (+)	
5-HT$_6$	Recombinant Endogenous (brain)	G$_s$ G$_s$	AC and PKA (+) AC and PKA (colliculus, striatum) (+)		gK$^+$ (striatum) (−) [90]	pERK (Fyn) (+)
5-HT$_7$ (4 splice variants)	Recombinant	G$_s$	AC and PKA (+)	G$_{12}$	RhoA and Cdc42 (+)	pERK (PKA and Epac) (+) pAkt (cAMP and Ca^{2+}) (+) p38 Kinase (PLCε) (+)
	Endogenous (brain)	G$_s$	AC and PKA (Hip) (+)	G$_{12}$ (Hip)	RhoA and Cdc42 (Hip) (+) gK$^+$ (striatum) (−) [90] gCation (globus pallidus) (+) [91]	pERK (Hip) (+) I$_H$ current (cAMP) (Hip, striatum) (+)

Table 1: Coupling patterns of multiple classes of 5-HT receptors(Millan et al., 2008).

3.6.2. Activation of PI3K/Akt pathway by 5-HT

Ligand-binding to 5-HT_{1A} can induce the activation of the PI3K/Akt/GSK3 pathway in primary cultures of hypothalamic (Cowen et al., 2005) or rhombencephalic neurons (Druse et al., 2005) and in the hippocampus *in vivo* (Polter et al., 2012). 5-HT_{1A}-induced activation of Akt is dependent on coupling with Gi proteins (Cowen et al., 2005; Hsiung et al., 2005). Activation of PI3K/Akt pathway by 5-HT_{1B} has been reported in transfected human neuroblastoma cells (Leone et al., 2000). Agonists of 5-HT_{2A} and 5-HT_{7A} receptors were reported to activate Akt in PC12 cells (Johnson-Farley et al., 2005).

3.7. Functions of 5-HT in the Central Nervous System

Brain serotonergic system is associated with the regulation of a variety of centrally controlled functions including feeding behavior (Breisch et al., 1976; Heisler et al., 2003; Leibowitz and Alexander, 1998), mood (Fernandez and Gaspar, 2012), social behavior (Kiser et al., 2012), sleep (Monti, 2011), thermoregulation (Cryan et al., 2000; Docherty and Green, 2010), cognition and memory (Buhot, 1997; Buhot et al., 2000), reward (Hayes and Greenshaw, 2011), locomotion (Jordan et al., 2008) and pain (Bardin, 2011). Additionally, impairment of serotonergic activity in the brain has been associated with a wide spectrum of psychiatric diseases including depression (Belmaker and Agam, 2008), schizophrenia (Remington, 2008) and anxiety (Fernandez and Gaspar, 2012).

3.7.1. Hypothalamic regulation of food intake

Serotonergic innervation from the median and/or dorsal raphe to the hypothalamus has been reported via several techniques many years ago (Azmitia and Segal, 1978; Beaudet and Descarries, 1979; Steinbusch, 1981). One of the principal actions of 5-HT in the hypothalamus is the regulation of food intake and energy homeostasis (Donovan and Tecott, 2013). Hypothalamic infusion of fenfluramine, a drug that increases the availability of 5-HT at the synapses (Rowland and Carlton, 1986), as well as $5\text{-HT}_{2C/1B}$ receptor agonists, initiate a robust anorexigenic effect (Vickers et al., 2000). The anorexigenic effect of 5-HT is primarily mediated by its action in the ARC (Donovan and Tecott, 2013). In addition, it has been demonstrated that microinjections

of 5-HT in the PVN, VMN and DMN nuclei induce suppression of food intake (Leibowitz and Alexander, 1998; Leibowitz et al., 1990; Paez and Leibowitz, 1993). These effects require the orchestrated serotonergic action via activation of numerous receptors (1A, 1B, 2B, 2C) in distinct neuronal subgroups, located in several hypothalamic nuclei (Sargent and Henderson, 2011; Vickers and Dourish, 2004).

a. Arcuate Nucleus

Serotonergic receptors are expressed in three neuronal subpopulations of the ARC, the orexigenic NPY/Agrp and RIP neurons, as well as the anorexigenic POMC neurons which receive and process peripheral and central signals in order to regulate energy homeostasis (Heisler et al., 2006; Hisadome et al., 2009; Rother et al., 2012; Xu et al., 2008; Yadav et al., 2009). Depending on the receptor that is stimulated, diverse cellular responses can occur, which can result in similar or opposed effects (Williams et al., 2011; Yadav et al., 2009).

i. 5-HT$_{2C}$

The anorectic effects mediated via this receptor were suggested by studies involving treatments with the agonist *m*-chlorophenylpiperazine (mCPP) and/or antagonists of 5-HT$_{2C}$ (Kennett and Curzon, 1991; Kitchener and Dourish, 1994). The importance of this receptor in feeding suppression was further reported in 5-HT$_{2C}$ null mice which exhibited increased food intake and body weight in comparison to wild type littermates (Tecott et al., 1995). Further, the role of 5-HT$_{2C}$ in the regulation of energy balance was described in mice expressing a mutated form of the receptor. These animals displayed not only hyperphagia and obesity but also late onset leptin resistance, insulin resistance and glucose intolerance (Nonogaki et al., 1998).

5-HT$_{2C}$ receptor is expressed in up to 80% of POMC neurons in the ARC (Heisler et al., 2002). The expression of 5-HT$_{2C}$ in POMC cells is required for the maintenance of energy homeostasis and the feeding suppression efficacy of dexfenfluramine (Heisler et al., 2002; Xu et al., 2008; Xu et al., 2010b). Moreover, hepatic insulin sensitivity is regulated by 5-HT$_{2C}$ activity in POMC neurons (Xu et al., 2010a). Stimulation of these

receptors by mCPP induces a PLC-dependent transient receptor potential C (TRPC) channel activation and inhibition of the K^+ M-current, which result in depolarization and increased excitability of the POMC neurons (Roepke et al., 2012; Sohn et al., 2011) (see Chapter 2, figure 5).

ii.   5-HT$_{1B}$

The involvement of 5-HT$_{1B}$ receptor in feeding behavior was first suggested after antagonist-induced reduction of the anorectic effects of dexfenfluramine (Grignaschi and Samanin, 1992). Mice with genetic deletion of this receptor were neither hyperphagic nor obese but they displayed insensitivity to the effects of dexfenfluramine on feeding behavior in comparison to wild type animals (Lucas et al., 1998).

5-HT$_{1B}$ receptors are expressed in the orexigenic NPY/Agrp neurons of the ARC, which form somatic and dendritic synapses with serotonergic axons (Heisler et al., 2006). Activation of these G$_i$-coupled receptors with the selective agonists CP94253 and CP93129 induces hyperpolarization of NPY/Agrp neurons and inhibits their spontaneous firing and neurotransmitter release by an unknown yet mechanism (Heisler et al., 2006). Consequently, Agrp-mediated inhibition of MC4Rs is attenuated and leads to food intake suppression (Heisler et al., 2006) (see Chapter 2, figure 5).

iii.   5-HT$_{1A}$, 5-HT$_{2B}$

5-HT$_{1A}$ receptor is expressed in the ARC Agrp/NPY and POMC neurons (Collin et al., 2002) and its activation can initiate orexigenic effects as it has been demonstrated with the use of agonists such as 8-hydroxy-2-(di-N-propylamino)tetralin (8-OH-DPAT) (Cooper et al., 1988; Gilbert et al., 1988). Recently, it has been demonstrated that mice with a genetic deletion of the 5-HT$_{1A}$ receptor in POMC neurons exhibit hypophagia and reduced body weight, suggesting an orexigenic action of this receptor (Yadav et al., 2011). Similarly, 5-HT$_{2B}$ deletion from POMC neurons resulted in feeding suppression, demonstrating an orexigenic effect of this receptor (Yadav et al., 2009).

iv.   5-HT$_{1F}$

A study reported that 5-HT reduces the excitability of RIP-Cre neurons in mice, and that this inhibition may be due to 5-HT$_{1F}$ receptors (Hisadome et al., 2009).

b.   Ventromedial Nucleus

The VMN receives dense serotonergic innervation from the raphe (Steinbusch, 1981; Willoughby and Blessing, 1987). 5-HT infusion inside the VMN induces a decrease in food intake and body weight (Fetissov and Meguid, 2010). Moreover, it was demonstrated that direct mCPP perfusion in the VMN suppresses food intake (Hikiji et al., 2004).

3.7.2. Hippocampal action of 5-HT and depression
a.   The serotonin deficiency hypothesis of depression

The serotonergic hypothesis of depression was first reported in the late 60's, based on the role of serotonergic deficiency in the physiopathology of this disorder (Coppen, 1967). This theory relies on a wide range of biochemical studies regarding the brain serotonergic function of depressed patients (Cowen, 2008). Moreover, this notion is supported by the antidepressant effects of increased 5-HT synaptic bioavailability caused by MAO inhibitors (Feldstein et al., 1964) or tricyclic antidepressants (Reznik Off, 1960), which decrease 5-HT oxidation and inhibit its neuronal uptake, respectively (Berton and Nestler, 2006). Additionally, the production and anti-depressive action of the selective serotonin reuptake inhibitors (SSRIs) reinforced even more the serotonergic theory of depression.

More recent studies further confirmed this notion by using brain imaging techniques such as positron emission tomography (PET) (Dhaenen, 2001; Miller et al., 2009; Sullivan et al., 2009).

Type of Drug	Mechanism of action	Examples
Tricyclic antidepressants (TCAs)	Inhibition of mixed noradrenaline and serotonin reuptake	Imipramine, desipramine
Selective serotonin reuptake inhibitors (SSRIs)	Inhibition of serotonin-selective reuptake	Fluoxetine, citalopram
Noradrenaline reuptake inhibitors (NRIs)	Inhibition of noradrenaline-selective reuptake	Atomoxetine, reboxetine
Serotonin and noradrenaline reuptake inhibitors (SNRIs)	Inhibition of mixed noradrenaline and serotonin reuptake	Venlafaxine, duloxetine
Monoamine oxidase inhibitors (MAOIs)	Inhibition of monoamine oxidase A (MAO-A)	Tranylcypromine, phenelzine

Table 2: Antidepressant drugs which target the serotonergic system.

b. 5-HT innervation of hippocampal neurons

Serotonergic projections that innervate dorsal hippocampus predominantly originate from the median raphe whereas the ventral hippocampal formation is innervated to greater extend by the median raphe (Mokler et al., 1999). Among the different areas of the dorsal hippocampus, the highest density of 5-HT axons was detected in *Ammon's horn* 3 (CA3), second highest in the *dentate gyrus* (DG) and the lowest in *Ammon's horn* 1 (CA1) (Mamounas et al., 1991). In the DG, fine serotonergic axons are found in the hilus and the molecular layer, but are almost absent from the granular area. However, beaded 5-HT terminals are extremely dense in the subgranular zone and less dense between the granule and molecular layers (Mamounas et al., 1991). The stratum radiatum and stratum oriens of the CA3 comprise intermingled beaded and fine serotonergic terminals whereas the layer of pyramidal cell bodies are almost lacking serotonergic axons (Mamounas et al., 1991). In the CA1, a network of fine 5-HT axon endings is detected in all areas except that of pyramidal cell bodies where the density is very low (Mamounas et al., 1991).

c.   Hippocampal 5-HT signaling and depression

Almost all the serotonin receptors are highly expressed in the hippocampus pre- and postsynaptically (Berumen et al., 2012) and, similarly, molecules that underlie its production (TPH) (Sakowski et al., 2006), uptake (SERT) (Owashi et al., 2004) and degradation (MAO) (Jahng et al., 1997) are present in this area.

Among all the 5-HT receptors, 5-HT_{1A} is the most extensively studied for its association with depressive and anxiety syndromes via its action in the hippocampus (Savitz et al., 2009). Presynaptic 5-HT_{1A} autoreceptors induce an inhibitory effect in serotonergic neurons that innervate the hippocampus (Chaput et al., 1986). Postsynaptically, the 5-HT_{1A} receptors are found in high densities in the CA1, CA3 and the DG (DG>CA1>CA3) (Barone et al., 1994; Verge et al., 1986). 5-HT action via these heteroreceptors induces hyperpolarization in CA1 and CA3 pyramidal neurons (Beck et al., 1992; Pugliese et al., 1998), LTP in the DG by GABAergic interneuron inhibition (Sanberg et al., 2006), cell proliferation, neurogenesis and survival in the subgranular zone of the DG (Huang and Herbert, 2005; Radley and Jacobs, 2002; Santarelli et al., 2003) as well asneuroprotection (Shibata et al., 1992). Besides the canonical cAMP pathway, hippocampal 5-HT_{1A} receptors can also activate other signaling pathways such as the PI3K/Akt/GSK3 and the MAPK pathways (Polter and Li, 2010). 5-HT_{1A} receptor agonists 8-OH-DPAT and azapirones induce anti-depressive effects as reported by the forced swim test (Wieland and Lucki, 1990) and the tail suspension test (Miyata et al., 2004), while the antagonists fail to affect depressive behaviors but block the antidepressant effects of desipramine and fluoxetine in the forced swim test (FST) and the tail suspension test, respectively (Detke et al., 1995; Miyata et al., 2004). Moreover, total 5-HT_{1A} knockout mice (Ramboz et al., 1998) and mice lacking only 5-HT_{1A} presynaptic autoreceptors (Bortolozzi et al., 2012) both display decreased depressive-like effects in the FST whereas over-expression of postsynaptic receptors had no effect in the FST immobility time but improved the antidepressant effects of the SSRI citalopram (Gunther et al., 2011). Thus a model of re-expression of this receptor only in the

serotonergic neurons of KO animals is needed in order to clearly understand the role of auto- and heteroreceptors in the antidepressant functions of 5-HT$_{1A}$.

Hippocampal action of 5-HT$_{1B}$ receptors plays an important role in depression despite its presence in low densities in this region (DG>CA1>CA3) (Verge et al., 1986). The effects of 5-HT$_{1B}$ autoreceptor activation involve the attenuation of 5-HT release in the ventral hippocampus as reported by SSRI treatment (e.g. paroxetine, fluoxetine) of KO mice for this receptor versus control mice (Gardier et al., 2003; Malagie et al., 2002).

5-HT$_{2A}$ receptor function has been associated with major depressive syndrome in human studies (Mintun et al., 2004; Sheline et al., 2004) but there is a lack of substantial animal studies to describe the mechanism of action. 5-HT$_{2A}$ receptor localization in hippocampus is mainly identified in the pyramidal cell layer of CA1/3 and in the granular layer of the DG (Li et al., 2004a).

5-HT$_{2B}$ receptor was recently reported to be necessary for both chronic and acute antidepressant effects of SSRIs (e.g. paroxetine and fluoxetine), with the use of agonists and KO mice for this receptor. It was suggested that these effects occur by increasing 5-HT release in the brain, including the hippocampus, acting as an activating autoreceptor on the serotonergic neurons of the raphe, wherein the expression of the receptor was demonstrated for the first time (Diaz et al., 2012). In the hippocampus it is expressed in astrocytes and its activation by the agonist alpha-methyl 5-HT results in increased intracellular Ca^{2+} levels which is blocked by the antagonist of 4-(4-fluorobenzoyl)-1-(4-phenylbutyl)-piperidine (Sanden et al., 2000).

5-HT$_{2C}$ receptor is localized in higher density in the pyramidal layers of CA1 and CA3 and the polymorph layer of the DG (Clemett et al., 2000). Antagonist infusion in the ventral hippocampus augmented the effect of the SSRI citalopram in the 5-HT release by an indirect mechanism which could involve GABAergic neurons (Cremers et al., 2007). Moreover, a selective 5-HT$_{2C}$ agonist administration results in reduced depressive-like behavior of rats as measured by the immobility time in the FST (Rosenzweig-Lipson et al., 2007). Isoforms that occur after alternative mRNA editing of the receptor introduce either more (isoleucine, asparagine, isoleucine, INI) or less (valine, glycine, valine, VGV)

depressive behavior in the FST, demonstrating the importance of the editing process in behavior (Mombereau et al., 2010).

5-HT$_3$ receptor in the rat hippocampus is expressed mainly in interneurons of the posteroventral CA1, in a region that receives significant serotonergic innervation (Tecott et al., 1993). 5-HT$_3$ KO mice exhibit increased immobility time in the FST, suggesting an antidepressant action of the receptor (Bhatnagar et al., 2004).

5-HT$_4$ receptor agonists induce rapid antidepressant effects in rats measured by the decreased immobility time and sucrose consumption in the FST and the sucrose preference test (SPT), respectively. Further, agonist administration increases neurogenesis and CREB phosphorylation in the hippocampus (Lucas et al., 2007; Pascual-Brazo et al., 2012). *In situ* hybridization revealed stronger expression in the granule layer of the DG, followed by the pyramidal cell layer of CA1 and CA2 areas (Vilaro et al., 2005).

5-HT$_5$ receptor is expressed in the rat hippocampus but it has not yet been clearly associated to depressive behavior (Oliver et al., 2000).

5-HT$_6$ receptor immunoreactivity is highly dense in the molecular layer of the DG and the strata oriens and radiatum of the CA1 (Gerard et al., 1997). Infusion of the 5-HT$_6$ receptor agonist EMD 386088 in the hippocampus of rats induces antidepressant-like effects such as decreased immobility time in the FST, an effect that is inhibited by the selective 5-HT$_6$ receptor antagonist SB-399885 (Nikiforuk et al., 2011).

5-HT$_7$ receptor and its mRNA are strongly detected in the pyramidal layers of CA1 and CA3 and the subgranular zone of the DG, as shown by immunohistochemistry and *in situ* hybridization (Neumaier et al., 2001). Administration of the 5-HT$_7$ receptor selective antagonist SB-258719 or the genetic deletion of the receptor induces antidepressant-like effects in the FST (decreased immobility time) (Guscott et al., 2005). Moreover, 5-HT$_7$ receptor heterodimerization with 5-HT$_{1A}$ receptor in hippocampal neurons attenuates the 5-HT$_{1A}$-mediated activation of GIRK channels (Renner et al., 2012).

CHAPTER 4

Leptin

4.1. General

Leptin, an adipocyte-secreted cytokine, regulates energy homeostasis and food intake, in addition to other physiological functions such as reproduction. It was named after the Greek word for thin, "λεπτός" (leptos), due to its role in acting against obesity.

The characterization of two mouse strains at the Jackson Laboratory, the obese and hyperphagic mice (ob/ob) by George Snell and his colleagues (Ingalls et al., 1950) and the diabetic (db/db) mice by Doug Coleman (Coleman, 1978), prepared the field for the discovery of leptin. The hypothesis of Douglas L. Coleman that the mutated gene causing obesity in the ob/ob mice encodes a circulating hormone which regulates body weight and that the *db* gene encodes the receptor of this hormone (Grayson and Seeley, 2012) was confirmed after cloning of these genes by the research group of Friedman (Zhang et al., 1994) and a group at Millennium Pharmaceuticals (Tartaglia et al., 1995) respectively .

4.2. Production, Secretion and Degradation

The adipocytes of the white adipose tissue are the main source of leptin production and secretion into the blood stream in levels which positively correlate to the body fat. Leptin secretion is regulated by hormones such as insulin, TNFα, NPY and glucocorticoids, by nutrients such as amino acids, lipids and glucose, and by neurotransmitters of the sympathetic nervous system (Lee and Fried, 2009). Leptin mRNA has also been detected in brown adipose tissue, placenta, ovaries, stomach, skeletal muscle, pituitary gland, mammary epithelial cells and liver (Margetic et al., 2002). Depletion of leptin takes place also in adipocytes via proteasomal and lysosomal action and it is regulated by nutrient levels and insulin (Lee and Fried, 2006).

4.3. Gene, Biosynthesis and structure

The human leptin is a 16 kDa (167 aa) protein which has a four-helix structure and belongs to the helical cytokine superfamily (Zhang et al., 1997). It is encoded by the gene *Lep* which is located at the genetic locus 7q31.3 (Zhang et al., 1994).

4.4. Leptin Signaling

4.4.1. Leptin Receptor (LepR/ObR)

The leptin receptor (LepR) belongs to the super family of class I cytokine receptors which includes receptors such as IL6, glycoprotein 130 (gp130), the leukemia inhibitory factor receptor alpha (LIF-R) and the ciliary neurotrophic factor (CNTF) receptors (Tartaglia et al., 1995).

The LepR gene is located at the genetic locus 1p31, 46.96 cM interval of chromosome 4, 5q33 in human, mice or rats, respectively (NCBI-Gene Database). There are six isoforms that occur upon alternative splicing or ectodomain shedding in rats, five in mice and four in humans, identified as LepRa, LepRb, LepRc, LepRd, LepRe (not in humans) and LepRf (only in rats) (Figure X) (Ge et al., 2002; Lee et al., 1996; Wauman and Tavernier, 2011). LepRb, the isoform with the long cytoplasmic domain, is the main isoform for the transduction of leptin signaling pathways. In contrast to the short isoforms, LepRa,c,d,e,f, LepRb is detected in low concentrations in peripheral tissues including adipose tissue, liver and muscles. However, in the hypothalamus, LepRb is highly expressed whereas the levels of short forms are low (Harwood, 2012).

During resting state, LepRb is present at the cellular membrane as a homodimer (Devos et al., 1997). The extracellular domain of the receptor contains two cytokine homology regions 2 (CHR2), an immunoglobulin-like domain (IgD) and two membrane-proximal fibronectin type III (FNIII) domains. Leptin epitope II binds to the CRH2 of one LepR and epitope III binds to the IgD of another LepR. This suggests that a LepR homodimer binds with 2 leptin molecules to form a complex with a 2:2 stoichiometry. These interactions lead to a change in the conformation of membrane-proximal regions which promotes phosphorylation of intracellular substrates (Mancour et al., 2012).The

intracellular domain of LepR is constitutively associated with Janus kinase 2 (JAK2), JAK2-binding protein SH2B and Src family kinases (SFKs) (Ghilardi and Skoda, 1997; Jiang et al., 2008).
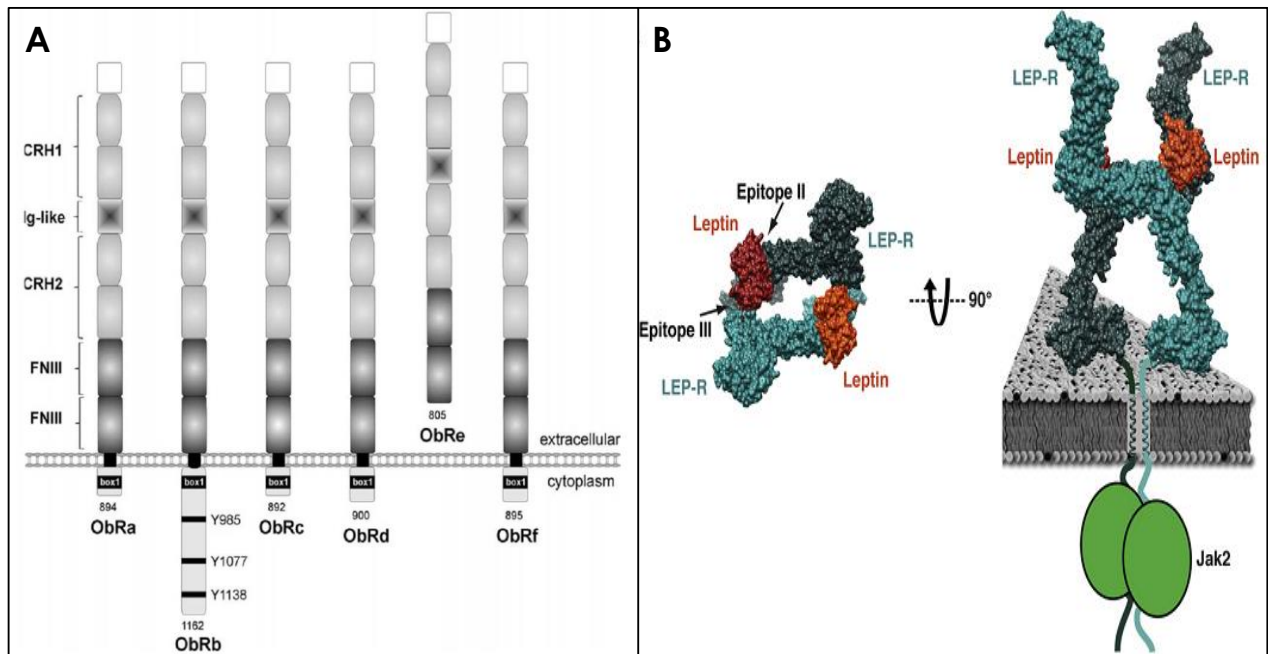

Figure 7: A. Leptin receptor isoforms in rats (from Wauman and Tavernier, 2011) B. Leptin/LepRb signaling architecture (from Mancour et al., 2012).

4.4.2. Substrates

i. Janus kinase 2 (JAK2)

JAK2 belongs to the JAK family of non-receptor kinase of 120-130 kD which also includes JAK1, JAK3 and TYK2. JAK1,2 and TYK2 are ubiquitously expressed and JAK3 expression is restricted to hematopoietic cells (Ihle, 1995).

Leptin/LepR binding results in transphosphorylation and transactivation of JAK2. Upon activation, JAK2 phosphorylates three Tyr residues of the intracellular domain of LepR. Phosphorylated Tyr^{985}, Tyr^{1077} and Tyr^{1138} residues serve as docking sites for SH2 domain-containing phosphatase 2 (SHP2), STAT5 and STAT3, respectively. Binding of these proteins to the receptor promotes their phosphorylation by JAK2 (Coppari and Bjorbaek, 2012). $Tyr^{813/1007/1008}$ phosphorylation of JAK2 occurs by autophosphorylation or by SFKs and SH2B and leads to full activation of JAK2 (Jiang et al., 2008).

ii. Signal transducer and activator of transcription 3/5 (STAT3/5)

STAT3 and STAT5 (750 to 850 amino acids) are members of the STAT family which contains seven isoforms, namely STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6.

Upon leptin/LepR binding, STAT3 undergoes Tyr^{705} and Ser^{727} phosphorylation, dimerizes and translocates into the nucleus as a homodimer where it regulates gene expression and mediates the effects of leptin (Santos and Costa-Pereira, 2011).

Tyr^{1077} phosphorylation of LepR results in phosphorylation of both STAT5 isoforms (A and B) at Tyr^{694} by JAK-2. Upon activation, STAT5 dimerizes and translocate to the nucleus in a mechanism similar to STAT3 (Gong et al., 2007).

iii. SHP2

SHP2 (or PTPN11) is a class 1 classical non-receptor PTP and contains 2 Src homology 2 domains in the N-terminal and one PTP domain in the C-terminal (Neel et al., 2003). Phosphorylated Tyr^{985} residue of LepR serves as docking site for SHP2 binding and activation. Mutation of Tyr^{985} to Phe blocks LepR/SHP2 binding and enhances STAT3 activation by leptin (Carpenter et al., 1998).

The knock-down of SHP2 in neurons enhances leptin-dependent JAK2/STAT3 activation whereas ERK activation was impaired revealing a bidirectional role of SHP2 in leptin signaling regulation (Zhang et al., 2004).

iv. Src family kinases (SFKs)

SFK family comprises 11 members (Blk, Brk, Fgr, Frk, Fyn, Hck, Lck, Lyn, Src, Srm, and Yes). SFKs are also expressed in leptin-targeted tissues (Roskoski, 2004). Leptin-dependent activation of SFKs contributes, at least partially, to the JAK2-independent effects of leptin. Besides, these kinases can activate JAK/STAT and MAPK/ERK pathways (Jiang et al., 2008).

v. SH2B

SH2B proteins are adaptor proteins that are involved in the signaling pathways of JAK kinases and many receptors such as insulin, IGF-I, nerve growth factor (NGF), brain-

derived neurotrophic factor (BDNF). SH2B family comprises three members of 548-756 amino acids: SH2B1 (or PSM), SH2B2 (or APS) and SH2B3 (or Lnk). They all share similar protein regions consisting of SH2 domain, PH domain, several proline-rich regions and a dimerization domain (Maures et al., 2007).

In the absence of stimulation, SH2B1 is constitutively associated with inactive JAK2 via the non-SH2 domains and inhibits its activity. The phosphorylation of JAK2 on Tyr[813] in response to leptin promotes the binding of the pY residue to the SH2 domain of SH2B1 and critically increases the interaction between the two proteins (Li et al., 2007). The Tyr[813]/SH2B1 interaction enhances leptin-induced JAK2 activity. SH2B1 recruits IRS1 and/or IRS2, creating a JAK2/SH2B1/IRS complex. This binding facilitates IRS tyrosine phosphorylation by JAK2 and prevents phosphatase-induced inactivation. IRS proteins phosphorylation in response to leptin activates the PI3K/Akt signaling pathways (Morris and Rui, 2009).

4.4.3. Leptin Signaling Negative Regulation

Leptin signaling is negatively regulated by the suppressor of cytokine signaling-3 (SOCS3) and phosphatases which interact directly with LepR (PTP1B, TC-PTP, and RPTP epsilon) or downstream molecules (SHP2 and PTEN).

a. Suppressor of cytokine signaling-3 (SOCS3)

SOCS3 is a member of the SOCS family which includes 8 members (SOCS1-7 and CIS) with similar structure containing a C-terminal SOCS-Box domain and a SH2 domain in the center. The SH2 domain serves as binding site to phosphotyrosine residues (Starr and Hilton, 1998). Leptin signaling activation induces the expression of SOCS3 (Bjorbaek et al., 1999). SOCS3 binds to and inhibits JAK2 (Bjorbaek et al., 1999), LepR (by Tyr[985] phosphorylation) (Bjorbak et al., 2000). SOCS3 deletion (Howard et al., 2004) and LepR mutation of Tyr[985] (Bjornholm et al., 2007) improve leptin sensitivity.

b. Protein tyrosine phosphatase 1-B (PTP1B)

PTP1B interacts with JAK2 and inhibits its activity by dephosphorylation (Cheng et al., 2002). Similarly, STAT3 gets dephosphorylated by PTP1B (Lund et al., 2005). PTP1B null mice show increased leptin sensitivity (Zabolotny et al., 2002).

c. T cell protein tyrosine phosphatase (TC-PTP)

TC-PTP dephosphorylates STAT3 and blocks its activity (ten Hoeve et al., 2002). Neuronal inhibition of this protein improves leptin signaling and double deletion of PTP1B and TC-PTP appears to increase leptin sensitivity through an additive effect (Loh et al., 2011).

d. Receptor protein tyrosine phosphatase epsilon (RPTPe)

RPTPe belongs to the receptor subgroup of PTPs. Leptin signaling activation induces tyrosine phosphorylation of RPTPe and this activation leads to interaction with and inhibition of JAK2 (Rousso-Noori et al., 2011).
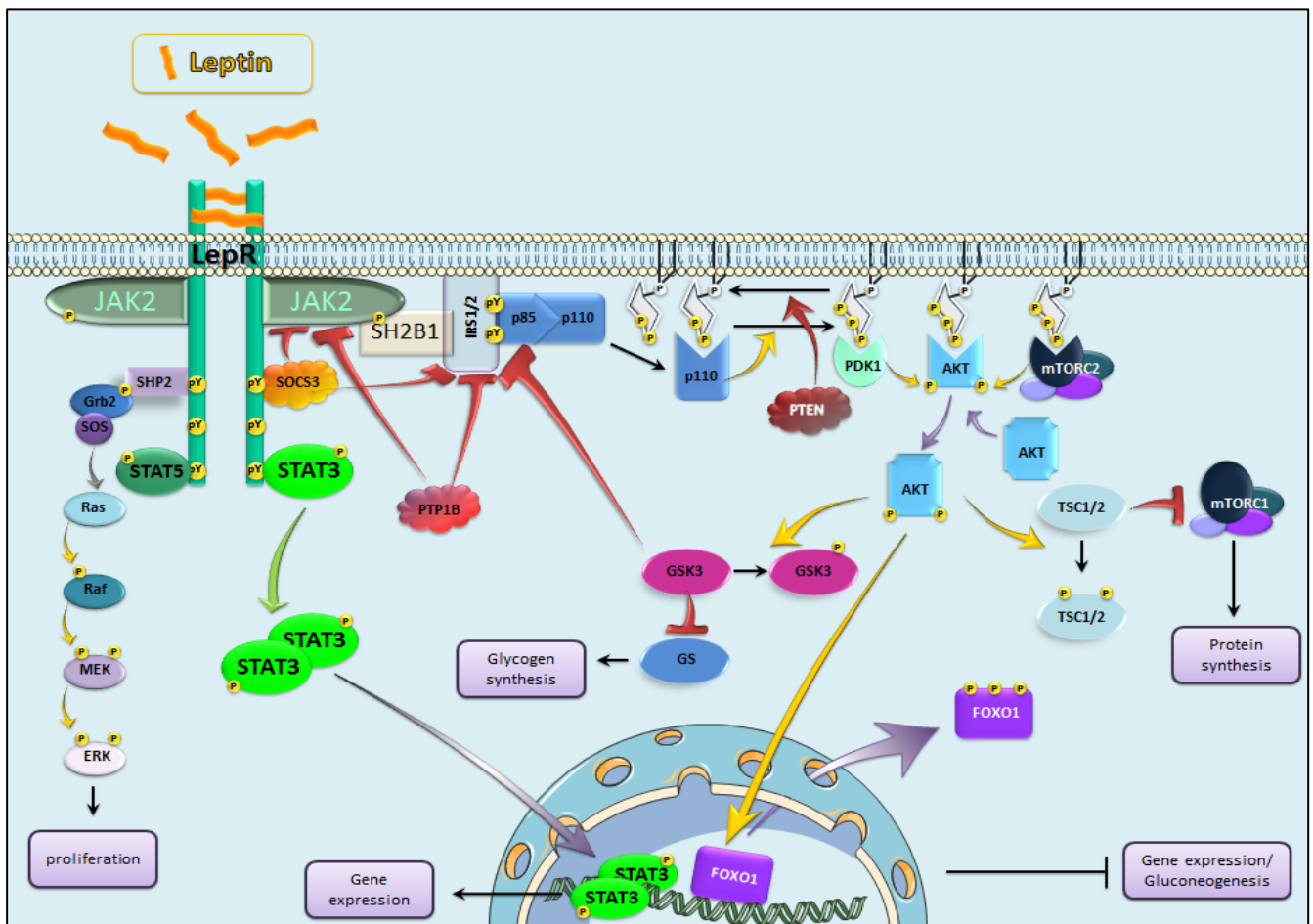

Figure 8: Leptin signaling pathway map.

4.4.4. Actions

a.   Periphery

Although the effects on regulation of body weight and energy homeostasis are mediated by the action of leptin in the brain, in peripheral tissues, leptin action is not redundant since it can induce various effects. (Guo et al., 2007).

In adipocytes, leptin action promotes lipid oxidation and lipolysis and inhibits fatty acid synthesis (Wang et al., 1999). In addition, increased leptin signaling activation in the adipose tissue prevented from diet-induced obesity and revealed a negative feedback regulation of leptin production by leptin (Wang et al., 2005). In the liver, leptin action decreases lipid levels in a PI3K- dependent mechanism (Huang et al., 2004). Moreover, leptin receptor over-expression in hepatocytes is protective against high fat diet-induced hepatic steatosis and hypertriglyceridemia (Lee et al., 2001). In skeletal muscle, leptin mediates fatty-acid metabolism via activation of 5'-AMP-activated protein kinase (AMPK) (Minokoshi et al., 2002). In pancreatic β-cells, leptin inhibits insulin expression and secretion, cell proliferation, cell size and apoptosis (Marroqui et al., 2012). LepR deletion from pancreas improves insulin production, glucose tolerance and insulin signaling (Morioka et al., 2007).

b.   Central nervous system

Leptin enters the CNS by crossing the BBB via a saturable transport system (Banks et al., 1996), which is facilitated by LepRa (Kastin et al., 1999), the most abundant leptin receptor isoform expressed in the BBB endothelial cells (Bjorbaek et al., 1998). On the other hand, the soluble receptor LepRe impairs the transport of circulating leptin in the brain (Pan et al., 2008).

After crossing the blood-brain barrier, leptin acts on diverse brain regions and circuits to regulate a wide range of physiological functions such as food intake, energy expenditure, thermogenesis, reproduction, lipid and glucose metabolism, cognition and behavior (Morrison, 2009). Specific neuronal populations in the hypothalamus,

hippocampus, brainstem and other brain nuclei are targeted by leptin which binds to LepRb expressed by these neurons and regulates their activity (Patterson et al., 2011).

i.   Hypothalamus

Leptin action in the hypothalamus regulates energy balance by acting in specific neuronal populations (table X). Several different hypothalamic nuclei have been identified to contain populations of neurons which express LepRb as well as projections of neurons that express LepRb (Patterson et al., 2011). The highest levels of LepR expression detected with IHC and ISH are found in the Retrochiasmatic area, the Arcuate nucleus (ARC), the Ventromedial nucleus (VMH), the Lateral hypothalamic area (LHA), the Dorsomedial nucleus (DMH) and the Ventral premammillary nucleus (Scott et al., 2009).

Region	Cell type	Food Intake	Body weight	Energy expenditure	Glucose homeostasis	References
ARC	POMC	no	yes	no	yes	(Balthasar et al., 2004)
ARC	NPY-AGRP	no	yes	no	no	(van de Wall et al., 2008)
ARC	NOS-1	yes	yes	yes	yes	(Leshan et al., 2012)
VMH	SF-1	yes	yes	yes	yes	(Dhillon et al., 2006)
LHA	neurotensin	no	yes	yes	no	(Leinninger et al., 2011)

Table 3: Leptin-targeted hypothalamic neurons which regulate energy homeostasis.

ii.   Hippocampus

Besides maintaining energy homeostasis and body weight, leptin's action in the brain plays an important role in cognitive function, behavior and mood as well as in protection against cognitive decline and stress. These brain functions are regulated, at least in part, through leptin action in the hippocampus (Lu, 2007; Morrison, 2009). In addition, hippocampal leptin action regulates food intake by inhibiting food related memories (Kanoski et al., 2011).

The expression levels of LepRb in the hippocampus are higher in the granular area of the dentate gyrus (DG) whereas in Ammon's horn (CA1-3) are quite low or undetectable

as evidenced by immunoreactivity and ISH (Scott et al., 2009). However, projections of LepRb expressing neurons have been found to be more dense in the CA1-3 regions than in the DG (Patterson et al., 2011).

Leptin action in the hippocampal neurons directly regulates synaptic functions and synaptic plasticity, neurogenesis and neuronal survival (Morrison, 2009).

Leptin positively (Oomura et al., 2006) and negatively (Moult et al., 2009) regulates the induction of long term potentiation (LTP) in CA1 hippocampal cells and leptin signaling in this region is necessary for this process (Li et al., 2002). In addition, it has been demonstrated that leptin regulates glutamatergic neurotransmission, via the AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid), NMDA (N-methyl-D-aspartate) and GluR1receptors, and modulates synaptic function (Durakoglugil et al., 2005; Irving et al., 2006; Moult et al., 2010; Shanley et al., 2001). Finally, leptin-induced PI3K signaling inhibits hippocampal neurons by activating large conductance Ca^{2+}-activated K^+ (BK) channels (O'Malley et al., 2005; Shanley et al., 2002a; Shanley et al., 2002b).

The subgranular zone (SGZ) of the dentate gyrus of the hippocampus is one of the three sites in the brain, along with subventricular zones of the lateral and third ventricle, where adult neurogenesis takes place (Hsieh, 2012; Lee et al., 2012). Leptin acts in the SGZ as a proliferation factor to enhance adult neurogenesis in vivo and in vitro via activation of STAT3 and Akt signaling pathways (Garza et al., 2008) as well as to restore stress-induced decrease of neural progenitor cell proliferation (Garza et al., 2012). A neuroprotective role of leptin in the hippocampus has been demonstrated to be mediated via JAK/STAT3, PI3K/Akt and MAPK/ERK1/2 signaling pathways (Guo et al., 2008; Zhang and Chen, 2008).

Finally, hippocampal action of leptin has been associated to anti-depressant like effects in behavioral tests such as forced swim test (FST), tail suspension test (TST) and sucrose (or saccharine) preference test (SPT). Brain infusion of leptin decreases depressive-like behavior in rats subjected to chronic stress, as addressed by FST and SPT, only when administered at the hippocampus (Lu et al., 2006). Mice lacking leptin

receptor in their dentate gyrus exhibit more depressive like behavior than control animals as evaluated with TST and SPT (Guo et al., 2012).

4.4.5. Leptin Resistance

Despite the fact that the term "leptin resistance" is widely used in an increasing number of publications, no universal, quantifiable or clinically useful term can be attributed (Myers et al., 2012). This term is used to describe a broad spectrum of conditions including hyperleptinemia (Frederich et al., 1995) or lack of responsiveness to leptin in inducing an anorexic effect and increasing energy expenditure (Tam et al., 2012). At the cellular level, "leptin resistance" is used for describing the impairment of leptin ability to activate a number of signaling pathways such as JAK2/STAT3 (Bjorbaek et al., 1999), PI3K/Akt (Benomar et al., 2005a) which is due to the increased action of negative modulators including SOSCS3 (Bjorbaek et al., 1999) and PTP1B (Benomar et al., 2009; Cheng et al., 2002).

CHAPTER 5

The PI3K/ Akt signaling pathway

5.1. General

The phosphatidylinositol 3-kinases **(**PI3K)/Akt signaling pathway is one of the most extensively studied intracellular signaling cascades and is involved in signal transduction of numerous tyrosine kinase receptors as well as several cytokine receptors.. Activation of the PI3K/ Akt pathway triggers a cataract of processes that control cellular functions including proliferation, growth, survival, migration, trafficking and metabolism. The dysfunction of PI3K/Akt signaling pathway has been reported in numerous diseases such as metabolic syndrome and cancers, revealing thus this signaling cascade as a potential therapeutic target (Bartholomeusz and Gonzalez-Angulo, 2012; Braccini et al., 2012; Chen et al., 2012b; Ciraolo et al., 2008; Knight et al., 2006; Vucenik and Stains, 2012)

5.2. The phosphatidylinositol 3-kinases

The phosphatidylinositol 3-kinases (PI3Ks) form a family of intracellular kinases that catalyze the phosphorylation of the membrane-associated phospholipid phosphatidylinositol and its products, the phosphoinositides (PtdIns), at the 3′‑hydroxyl group/ring. In turn, the products of this reaction activate downstream effector proteins and their respective signaling cascades. Consequently, PI3Ks regulate a number of functions including cell metabolism, polarity, proliferation and survival.(Di Paolo and De Camilli, 2006).

The eight different mammalian PI3K protein isoforms are divided in three Classes (I, II and III) according to their homology, structure and biochemical action. They all comprise common structural motifs including C2, helical and catalytic domains (Vanhaesebroeck et al., 2010).
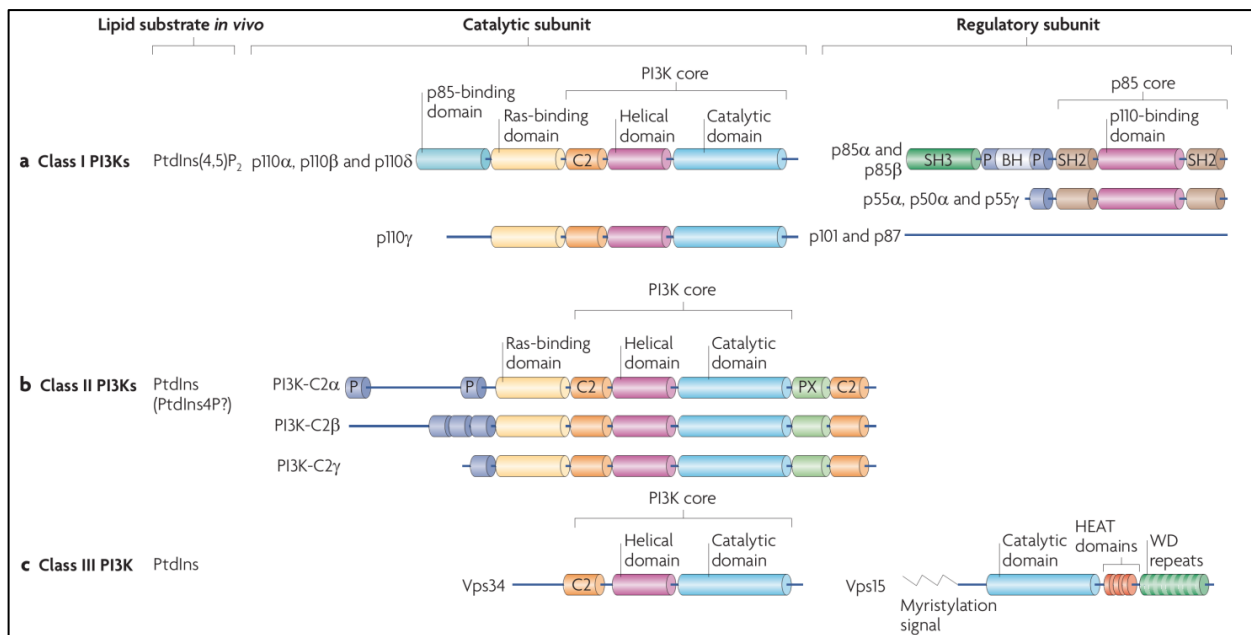
Figure 9: Structure of PI3K isoforms (Vanhaesebroeck et al., 2012).

5.2.1. Class I PI3Ks

The best characterized group of PI3Ks is the Class I and it is divided into two subgroups, I_A and I_B. The members of both subgroups catalyze the production of PtdIns(4,5)P_2 from PtdIns(3,4,5)P_3 (Hawkins et al., 1992; Vanhaesebroeck et al., 2010; Vanhaesebroeck et al., 2012).

a.   Class I_A

Class I_A PI3Ks are heterodimeric structures formed by one regulatory subunit (p85α, p85β, p55γ, p55α or p50α) and one catalytic subunit (p110α, p110β or p110δ) which catalyzes the production of PtdIns(3,4,5)P_3 from PtdIns(4,5)P_2 (Geering et al., 2007; Vanhaesebroeck et al., 1997). The p85α subunit contains two Src homology 2 (SH2) domains (nSH2 and cSH2) which serve as docking sites with very high affinity for phosphorylated tyrosine-containing motifs (pY) that belong to membrane receptors and their substrates or other membrane-proximal proteins (Felder et al., 1993; McGlade et al., 1992). A third SH2 (iSH2) domain interacts with the ABD and C2 domains of p110 (Dhand et al., 1994; Wu et al., 2009). In basal conditions, the p85 subunit stabilizes and blocks the catalytic action p110 (Yu et al., 1998). RTKs, GPCRs and their substrates are the main

type of receptors which interact with and activate Class I_A PI3Ks (Vanhaesebroeck et al., 2012). Upon p85 binding to the pY residues in the sequence context pYxxM (Songyang et al., 1993) of activated RTKs or adaptor proteins via its SH2 domains, the enzymatic activity of p110 is enabled due to abolition of the p85-mediated inhibition and the affinity of the p85/p110 complex to lipid membranes is increased (Burke et al., 2011; Shoelson et al., 1993; Zhang et al., 2011) The action of p85 can be inhibited by phosphorylation of two serine residues (Ser[361] and Ser[652]) at analogous sites on both SH2 domains of p85α (Foukas et al., 2004; Lee et al., 2011). Phosphorylation state of the Tyr[688] amino acid residue is also involved in the regulation of p85 and thus PI3K activity (Cuevas et al., 2001; Tsuboi et al., 2008). p110α is involved in the insulin signaling pathway (Knight et al., 2006) and plays an essential role in metabolism and growth due to increased interaction with and activation of IRS proteins (Foukas et al., 2006). The p110β subunit has been also demonstrated to be involved in the signal transduction of insulin and leptin (Al-Qassab et al., 2009) and to regulate metabolism and cell growth (Ciraolo et al., 2008). Finally, the p110δ subunit regulates many the development and activation of immune cells (So and Fruman, 2012).
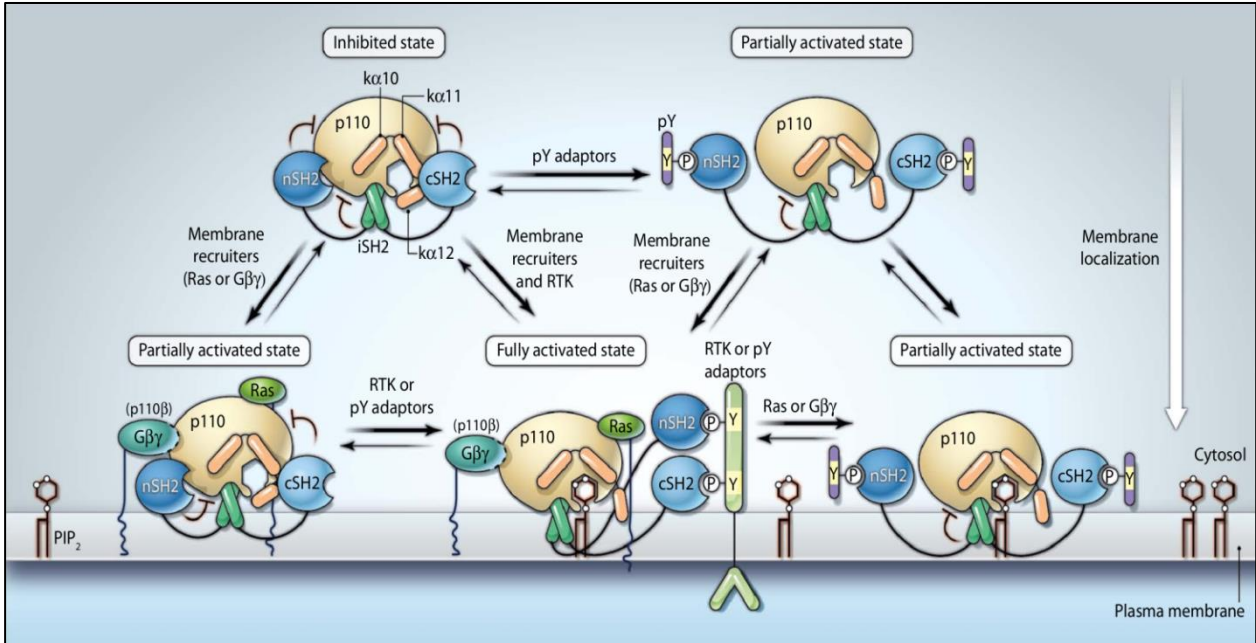

Figure 10: Class I PI3K activation and enzymatic action (Vadas et al., 2011).

b.   Class I_B

Class I_B PI3Ks contains the catalytic subunit p110γ which complexes with one of the regulatory subunits, p101, p84 or p87PIKAP which, in contrast to p85, do not contain SH2 domains (Engelman et al., 2006). Upon GPCR activation, the Gβγ subunits recruit to the cell membrane and activate the p101/p110γ complex (Brock et al., 2003; Stephens et al., 1997). The p101 subunit is required for full activation of p110γ by Gβγ and for directing its enzymatic preference towards PtdIns(4,5)P$_2$ (Maier et al., 1999; Stephens et al., 1997). Ras-GTP is essential for the membrane recruitment and activation of p87/p110γ but it also facilitates the activation of p110γ alone or p101/p110γ complex (Kurig et al., 2009). In cardiac cells p110γ is activated by phosphodiesterase 3B (PDE3B) and reduces β-adrenergic receptor density which results in heart failure, an effect that is blocked by PKA (Perino et al., 2011). In addition, PDE3B-induced activation of p110γ regulates angiogenesis (Wilson et al., 2011).

5.2.2. Class II PI3Ks

In contrast to Class I PI3Ks which are heterodimers, Class II PI3Ks (or PI3K-C2) are monomers. PI3K-C2α, PI3K-C2β and PI3K-C2γ are the three different isoforms characterized in mammals (Falasca and Maffucci, 2007; Vanhaesebroeck et al., 2010). These isoforms possess a Ras-binding domain and a Phox domain (Stahelin et al., 2006; Vanhaesebroeck et al., 2010). PI3K-C2α activation, which can be induced by several stimuli, including hormones, chemokines and cytokines, regulates glucose transport, insulin secretion, neuronal secretion and endocytosis (Falasca and Maffucci, 2007). Cell growth and survival, cell migration and K$^+$ channels activation are promoted by PI3K-C2β which can be activated by growth factors and phospholipids (Falasca and Maffucci, 2007; Falasca and Maffucci, 2012). So far there is no study that demonstrates a specific activator, product or mechanism of action for PI3K-C2γ (Falasca and Maffucci, 2007). PtdIns(3)P has been found to be the only product of Class II PI3Ks in cell cultures and *in vivo* (Domin et al., 2005; Falasca et al., 2007).
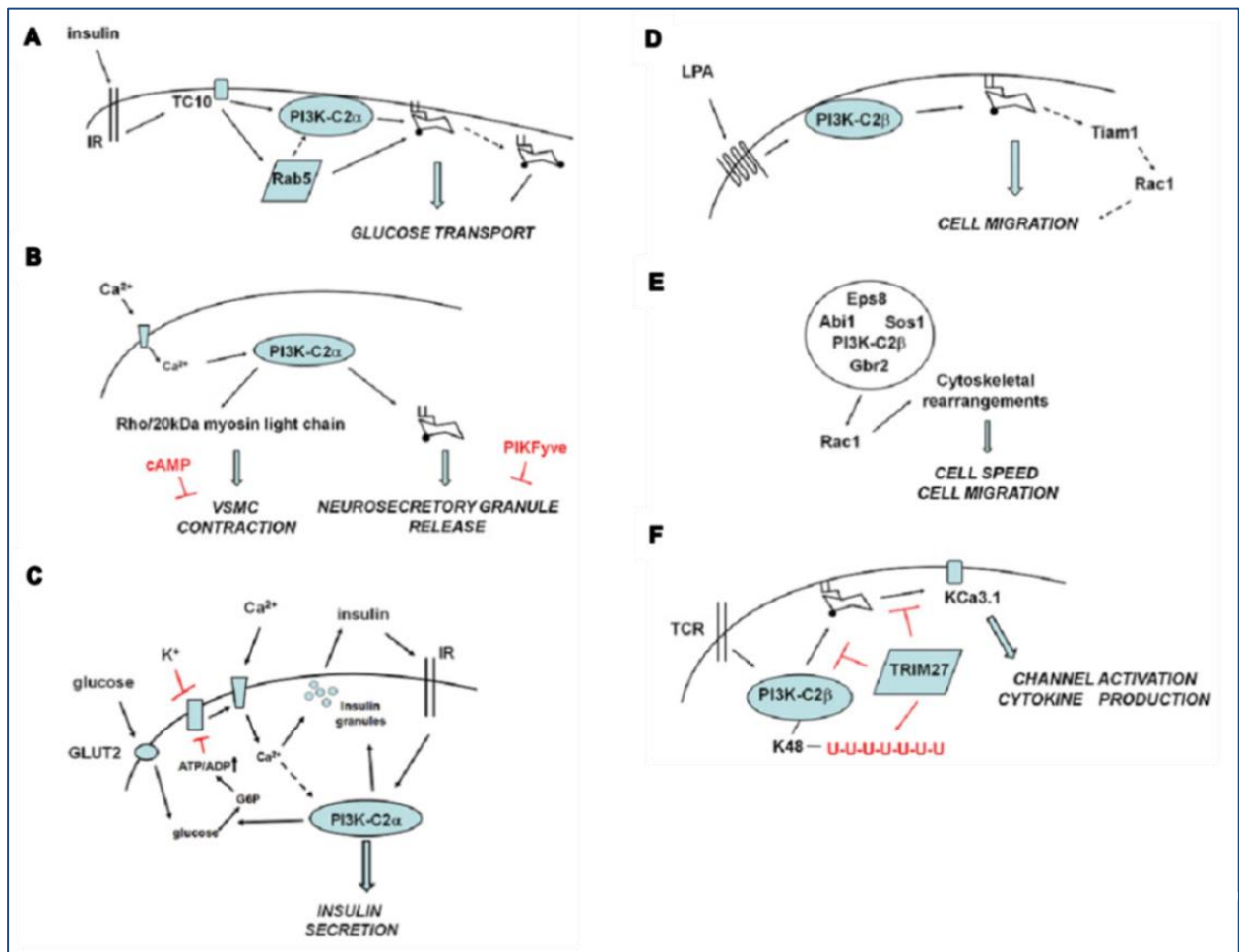
Figure 11: Activation and action of Class II PI3Ks. (Falasca and Maffucci, 2012).

5.2.3. Class III PI3Ks

This class contains only one isoform, the vacuolar protein sorting 34 (Vps34) (Backer, 2008). The mammalian homologous protein, hVps34, is ubiquitously expressed (Volinia et al., 1995). Vps34 is constitutively associated with and the Vps15 protein kinase which contains a membrane-targeting myristoylated domain (Stack et al., 1993). Vps34 signaling requires membrane-recruitment of other intracellular partners, including proteins with FYVE [Fab1p, YOTB, Vac1p, EEA1 (early endosomal antigen 1)] or PX (Phox homology domain) catalytic domains which bind PtdIns3P (Backer, 2008). The hVps34 interacts with beclin-1 together they create complexes which play an important role in autophagy and cell growth with other proteins such as UVRAG (Liang et al., 2006),

Atg14L and Rubicon (Matsunaga et al., 2009), MTM and mTORC1 (Blondeau et al., 2000; Fetalvero et al., 2013)as well as in endocytosis by interaction with Rab5 and Rab7 (Kinchen et al., 2008; Kitano et al., 2008).
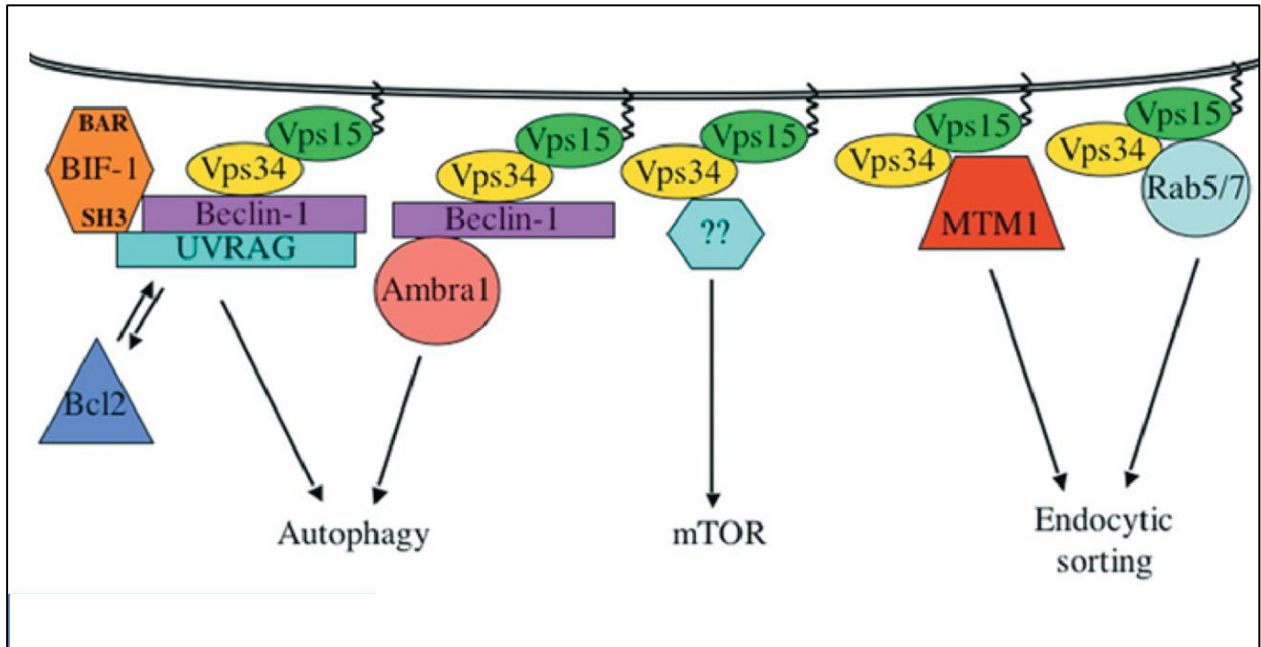

Figure 12: Class III PI3K complexes and action in mammals (Backer, 2008).

5.3. Phosphoinositides

Phosphoinositids (PtdIns) or Inositol phospholipids, derive from the phosphorylation of their precursor molecule phosphatidylinositol (Pins), or other PtdIns, on the inositol ring at positions 3, 4 and 5. The PI3K family enzymes catalyze this kind of phosphorylation which can be reversed by a number of phosphatases (INPP, MTM, PTEN, SHIP). These reactions give rise to seven phosphoinositide species (figure X). (Di Paolo and De Camilli, 2006; Vanhaesebroeck et al., 2012). The phosphorylated PtdIns, PtdIns-3-P, $PtdIns(3,4)P_2$ and $PtdIns(3,4,5)P_3$, serve as docking sites for downstream kinase that carry lipid-binding domains (i.e. the pleckstrin homology (PH) domain, the phox homology (PX) domain and the FYVE domain), which translocate to the cell membrane. This co-localization and binding leads to activation of these proteins and triggers their various downstream signaling cascades (DiNitto et al., 2003; Vanhaesebroeck et al., 2010).
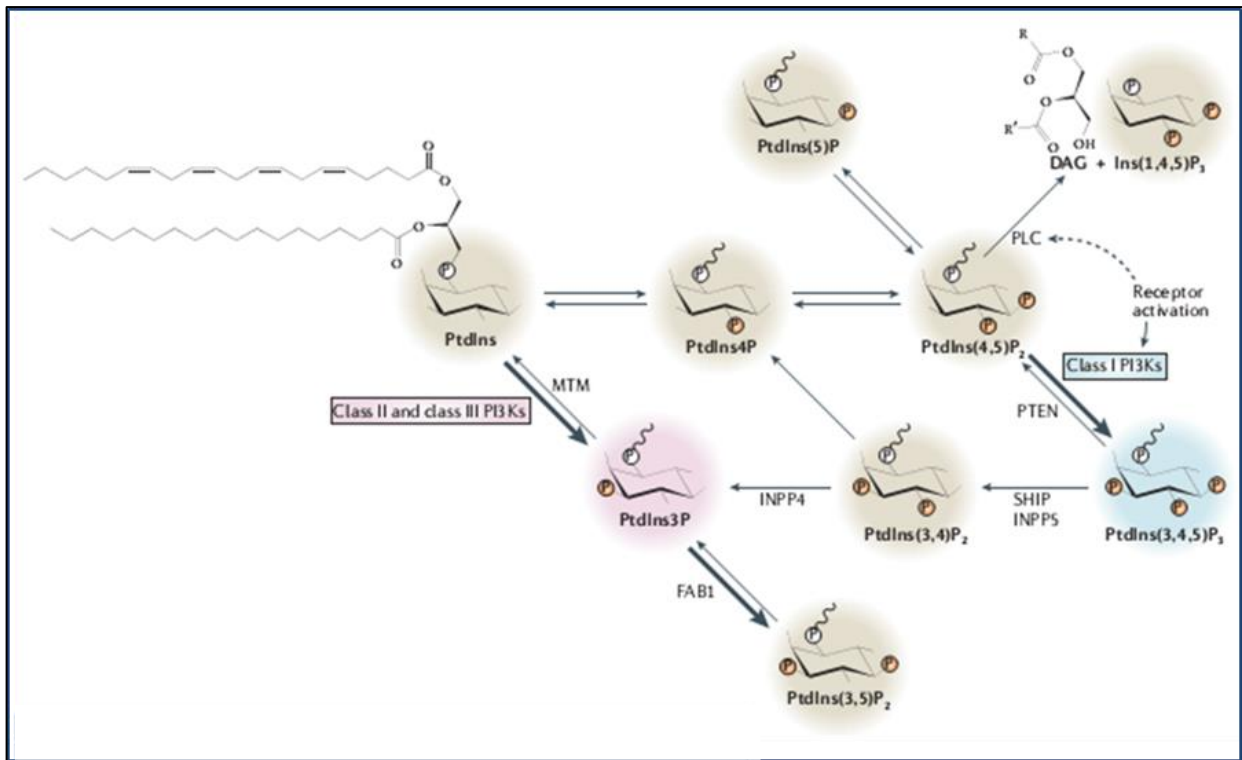
Figure 13: Action of PI3Ks and phosphatases on phosphoinositide transformation (Vanhaesebroeck et al., 2012).

5.4. Negative regulation of PI3Ks

5.4.1. Phosphatase and tensin homologue - PTEN

PTEN was first identified as the product of a tumor suppressor gene responsible for diverse types of cancer (Li et al., 1997). The product of this gene is a 403 amino-acid protein which contains an N-terminal domain (190 amino acids) which is homologous to the cytoskeletal protein tensin 1 and encloses a protein phosphatase domain with the motif HCXXGXXR (Lee et al., 1999). Moreover, a C-terminal C2 domain facilitates the interaction of PTEN with cell membranes (Lee et al., 1999). PTEN can dephosphorylate serine-, threonine- and tyrosine-phosphorylated peptides with higher specificity for acidic substrates (Myers et al., 1997). In addition, this phosphatase has been demonstrated to dephosphorylate the PtdIns(3,4,5)P3 specifically at position 3 on the inositol ring via the same enzymatic site opposing the activity of PI3K (Maehama and Dixon, 1998).

5.4.2. Inositol polyphosphate 4- phosphatase – INPP4

INPP4 interacts with the p85 subunit of PI3K to approach $PtdIns(3,4)P_2$ and dephosphorylate the D4 group to produce $PtdIns(3)P$ (Munday et al., 1999), a process that is essential for tumor suppression in several kinds of cancer such as breast cancers and ovarian cancers (Gewinner et al., 2009).

5.4.3. Inositol polyphosphate 5- phosphatases (5-ptases)

The 5-ptase family contains ten mammalian members which all contain a 300 amino acid catalytic domain. These enzymes dissociate the 5-position phosphate from the inositol ring of $PtdIns(4,5)P_2$, $PtdIns(3,4,5)P_3$ and/or $PtdIns(3,5)P_2$ (Ooms et al., 2009).

The Src homology 2-domain-containing inositol phosphatase or SHIP2 (also known as inositol polyphosphate 5'-phosphatase-like protein-1 or INPPL1) is a 142 kDa protein which contains an SH2 domain on the N-terminal, 5-ptase domain in the center and a proline rich domain (PRD) followed by a sterile alpha-motif (SAM) domain on the C-terminal (Suwa et al., 2010). SHIP2 dephosphorylates of $PtdIns(3,4,5)P_3$ and $Ins(1,3,4,5)P_4$ to negatively regulate PI3K-mediated activation of downstream pathways (Pesesse et al., 1998). SHIP2 has been shown to be an essential regulator of various pathologies including insulin sensitivity/ diabetes (Clement et al., 2001), obesity (Sleeman et al., 2005), neurodegeneration (Soeda et al., 2010), cancer (Wisniewski et al., 1999) and atherosclerosis (DeKroon et al., 2006).

Haemopoietic-specific 5-ptase (SHIP1) is a 145 kDa protein that has not only a highly homologous structure to SHIP2 (with the exception of the SAM domain) but also the same enzymatic function to hydrolyze $PtdIns(3,4,5)P_3$ and $Ins(1,3,4,5)P_4$ (Damen et al., 1996; Rohrschneider et al., 2000). SHIP1 action negatively regulates proliferation of haematopoietic cells to prevent different types of leukemia (Fukuda et al., 2005; Horn et al., 2004).

Inositol polyphosphate 5-phosphatase B (also known as Type II 5-phosphatase) or INPP5b and the 72 kDa inositol polyphosphate 5-phosphatase or INPP5e dephosphorylate $PtdIns(3,4,5)P_3$ and $PtdIns(4,5)P_2$ into $PtdIns(3,4,)P_2$ and $PtdIns(4)P$

respectively (Bielas et al., 2009; Jefferson and Majerus, 1995). Mutations of the INPP5E gene have been shown to cause different kinds of ciliopathies (Bielas et al., 2009).

5.5. Phosphoinositide dependent protein kinases

5.5.1. Akt/PKB protein kinase

The Akt/PKB protein kinase family belongs to the AGC group of protein kinases (named after PKA, PKG, and PKC). This serine/threonine kinase family is composed of three members in mammals: AKT1/PKBα (480 amino acids, MW: 55.7 kDa), AKT2/PKBβ (481 amino acids, MW: 55.8 kDa) and AKT3/PKBγ (479 amino acids, MW: 55.8 kDa). They share more than 80% common amino-acid sequence and they are products of three distinct genes, located in different chromosomes. All three isoforms have a PH domain on the N-terminal, a catalytic domain in the center and a hydrophobic domain on their C-terminal. This structure is conserved among species from fly, worm, mouse, to human. AKT1 and AKT2 are ubiquitously expressed and this expression is more remarkable in insulin targeted tissues. AKT3 expression is mainly observed in lipid-rich tissues including brain, testis, adipose tissue and liver. (Fayard et al., 2010; Hanada et al., 2004; Schultze et al., 2011).
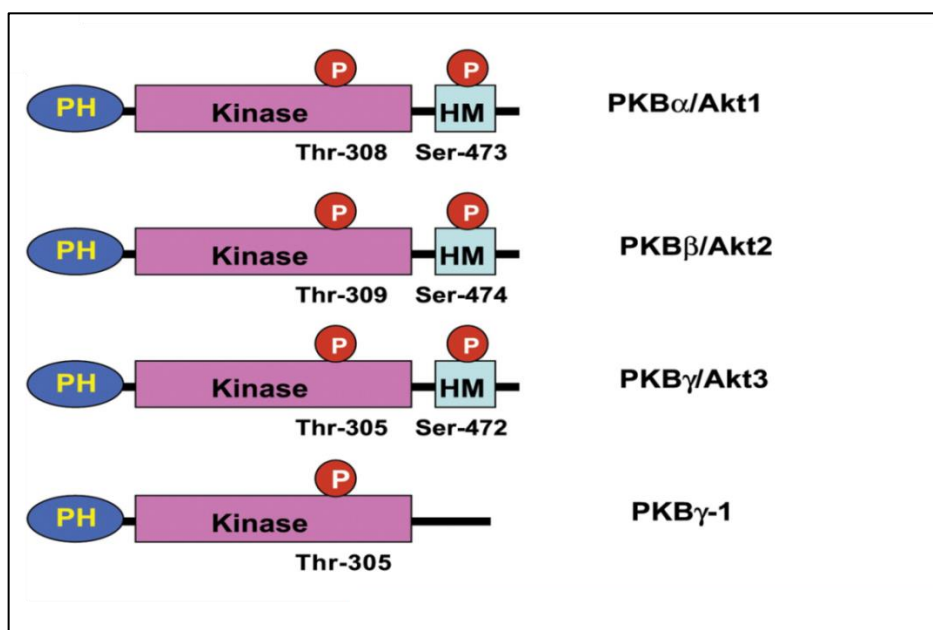

Figure 14: Protein structure of Akt isoforms (Hanada et al., 2004).

The Akt gene was first described as v-akt oncogene of the AKT8 murine retrovirus where it was first isolated (Staal et al., 1977) and ten years later the human AKT1 and AKT2 homologues were cloned by the same group. (Staal, 1987). Two other groups cloned the akt gene from human cells in 1991 and named it rac (related to the A and C kinases)(Jones et al., 1991) and PKB(protein kinase B)(Coffer and Woodgett, 1991). The last family member AKT3/PKBγ was cloned few years later (Brodbeck et al., 1999).

Akt is located downstream of PI3Ks. After translocation to the membrane, this kinase binds through its PH domain to PtdIns(3,4,5)P3 and PtdIns(3,4)P2. This binding facilitates the positioning of Akt in proximity to the membrane and its subsequent phosphorylation by upstream kinases (Calleja et al., 2007; Milburn et al., 2003). Both Akt1 phosphorylation sites, Thr^{308} and Ser^{473} (Thr^{309} and Ser^{474} for AKT2, Thr^{305} and Ser^{472} for AKT3), situated in the activation loop of the catalytic domain and the hydrophobic regulatory domain respectively, need to be phosphorylated in order to achieve full kinase activity and dissociation from the cell membrane (Alessi et al., 1996). $PtdIns(3,4,5)P_3$ and $PtdIns(3,4)P_2$ levels positively correlate with Thr^{308} and Ser^{473} phosphorylation respectively (Ma et al., 2008). Phosphorylation of the Thr^{308} residue is mediated by PDK1 (3-phosphoinositide-dependent protein kinase-1) which is also recruited to the membrane by PIP3 through its PH domain (Alessi et al., 1997). Multiple candidate PDK-2 (or S473K) kinases, such as DNA-PK, ATM and mTORC2, have been proposed to catalyze the phosphorylation of Akt (Fayard et al., 2005). Besides, its role in the phosphorylation and activation of Akt, mTORC2 has been reported to regulate Akt conformation and stability by phosphorylating the Thr^{450} site of the TM (turn motif) domain (Facchinetti et al., 2008; Sarbassov et al., 2005). Recently, a study has demonstrated that PI3K-mediated Akt phosphorylation on Ser^{473} is through direct activation of mTORC2 by $PtdIns(3,4,5)P_3$, indicating that mTORC2 is the most successful candidate to be called PDK2 (Gan et al., 2011).

Akt activation can be blocked by phosphatases either directly or indirectly. Protein phosphatase 2A (PP2A) acts directly on Akt and dephosphorylates both Thr^{308} and Ser^{473}leading to full inactivation of its kinase activity (Ugi et al., 2004). Phosphatase and

tensin homologue (PTEN) and SH2 domain-containing inositol polyphosphate 5-phosphatase (SHIP) prevent indirectly Akt activation by counter-acting on PI3K action. These two phosphatases dephosphorylate PIP3 and produce PI(4,5)P2 and PI(3,4)P2, respectively (Ooms et al., 2009; Song et al., 2012).

Following activation, Akt changes conformation, detaches from the cell membrane and translocates to the cytoplasm or the nucleus(Calleja et al., 2007). Consequently, Akt regulates numerous signaling molecules (see table 4) involved in a broad range of cellular functions in response to extracellular stimuli (Fayard et al., 2005). Indeed, upon phosphorylation Akt substrates regulate a large spectrum of cell processes including metabolism, glucose uptake, angiogenesis as well as cellular proliferation, survival and growth. Activation of each downstream protein can induce either a single specific effect or pleiotropic effect depending on the cell processes that it controls. In table 4 the list of the most well studied substrates is presented and their respective function/s.

Protein name	Phospho-residues	Protein effect	Cellular Function	Cellular Effect	References
FOXO (1,3,4,6)	T24, S256, S319	Inhibition	Transcription factors	Metabolism, proliferation, survival	(Tzivion et al., 2011)
GSK3α/β	S9/ S21	Inhibition	Kinase	Metabolism, proliferation, survival	(Manning and Cantley, 2007)
BAD	S99	Inhibition	Apoptotic protein	Survival	(Datta et al., 1997)
IKK	T23	Activation	Kinase	Inflammation, cell stress	(Ozes et al., 1999)
Mdm2	S166,S186	Activation	Ligase	Cell survival, proliferation	(Mayo and Donner, 2001)
TSC2	S939, T1462	Inhibition	mTORC blocker	Cell growth, proliferation	(Huang and Manning, 2009)
eNOS	S1177	Activation	Synthase	angiogenesis	(Dimmeler et al., 1999)
Casp9	S196	Inhibition	Protease	survival	(Cardone et al., 1998)

Table 4: Akt protein kinase substrates and their functions.

5.5.2. GSK3 protein kinase

Glycogen synthase kinase 3 has been named after its earliest discovered role as inhibitor of the glycogen synthase (Embi et al., 1980).This serine/threonine kinase exists in two isoforms, α and β, which share a 97% homology in the kinase domain. GSKα (51 kDa) and GSK3β (47 kDa) are ubiquitously expressed, with higher levels in the brain (Woodgett, 1990). In addition, GSK3α and β present similar structure and substrate preference and their function is not redundant as shown by gene deletion studies (Li and Jope, 2010).
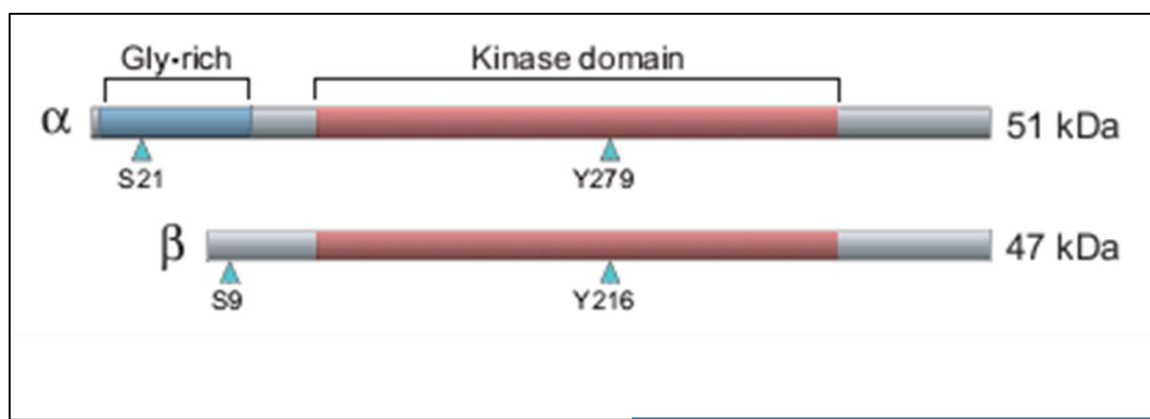

Figure 15: Protein structure of GSK3 isoforms (Doble and Woodgett, 2003).

GSK3 is constitutively active even in non-stimulated states and is inhibited when upstream signaling pathways are activated (Sutherland et al., 1993). GSK3 activity is regulated by phosphorylation, translocation, protein-protein interactions and substrate phosphorylation state (Doble and Woodgett, 2003). Phosphorylation of the Ser^9 residue of GSK3β or Ser^{21} residue of GSK3α, located at the N-terminal of the protein, is mediated by a number of upstream kinases such as Akt/PKB (Cross et al., 1995), PKA (Tanji et al., 2002), PKC (Fang et al., 2002), p70 ribosomal S6 kinase (Terruzzi et al., 2002), and p90 ribosomal S6 kinase (Roseweir et al., 2012). This phosphorylation leads to the inhibition of GSK3 and consequently to the production of glycogen (Doble and Woodgett, 2003). GSKβ, but not GSKα, is also deactivated by phosphorylation of the C-terminal Thr^{390} residue by p38 mitogen-activated protein kinase (Thornton et al., 2008). Finally, tyrosine

phosphorylation at Tyr^{279} of GSK3α and Tyr^{216} of GSK3β induces their activation (Buescher and Phiel, 2010).

GSK is involved in a wide range of biological processes as witnessed by numerous studies performed at the molecular and cellular levels. This occurs through the multiple substrates targeted by GSK3. The substrate motif recognized by GSK-3 is Ser/Thr–X–X–X–Ser/Thr-P (Roach, 1991). The effects of GSK3 on its substrates are mediated by either kinase activity or protein binding. Inhibitory phosphorylation occurs on primed (already phosphorylated, e.g. GS, eIF2B) or non-primed substrates (e.g PS-1). These effects regulate multiple cellular processes that are implicated in pathologies such as Type II Diabetes and Alzheimer's disease (Gao et al., 2012), mood disorders (Li and Jope, 2010) development and cancer (Rayasam et al., 2009).

Protein name	Phosphorylated residues	Protein effect	Protein Function	Cellular Effect	References
GS	S641, S645, S649, S653	Inhibition	Synthetic enzyme	Metabolism, proliferation, survival	(Rylatt et al., 1980)
IRS1	S332	Inhibition	Binding molecule	Metabolism, proliferation, survival	(Liberman and Eldar-Finkelman, 2005)
IRS2	S484/S488	Inhibition	Binding molecule	Metabolism, proliferation, survival	(Sharfi and Eldar-Finkelman, 2008)
PTEN	S362, T366	Activation, Stability	Phosphatase	Growth, proliferation, survival	(Al-Khouri et al., 2005)
PS1	S397	Inhibition	Protease	Proteolysis	(Twomey and McCarthy, 2006)
eIF-2B	S535, S539	Inhibition	Translation factor	Translation	(Mohammad-Qureshi et al., 2008)
5HT1B	-	Activation	GPCR	Neurotransmission	(Polter and Li, 2011)
TAU	>20	Misfolding and fibrilation	microtubule-associated	Microtubule stability	(Hanger et al., 2009)

Table 5: GSK3 protein kinase substrates and their functions.

RESULTS

ARTICLE 1

Hypothalamic serotonin–insulin signaling cross-talk and alterations in a type 2 diabetic model

Ioannis Papazoglou [a,b,1], Flavien Berthou [a,b,1], Nicolas Vicaire [a,b,1], Claude Rouch [c], Eirini M. Markaki [c], Danielle Bailbe [d], Bernard Portha [d], Mohammed Taouis [a,b,*], Kyriaki Gerozissis [a,b,e]

[a] CNRS, Center of Neurosciences Paris-Sud, UMR 8195, Orsay F-91405, France
[b] Univ Paris-Sud, UMR 8195, Orsay F-91405, France
[c] CNRS UMR 7059, Univ Paris 7, Paris F-75275, France
[d] B2PE, BFA, Univ Paris-Diderot/CNRS EAC4413, Paris F-75205, France
[e] INSERM, Orsay F-91405, France

ARTICLE INFO

ABSTRACT

Serotonin and insulin are key regulators of homeostatic mechanisms in the hypothalamus. However, in type 2 diabetes, the hypothalamic responsiveness to serotonin is not clearly established. We used a diabetic model, the Goto Kakizaki (GK) rats, to explore insulin receptor expression, insulin and serotonin efficiency in the hypothalamus and liver by means of Akt phosphorylation. Insulin or dexfenfluramine (stimulator of serotonin) treatment induced Akt phosphorylation in Wistar rats but not in GK rats that exhibit down-regulated insulin receptor. Studies in a neuroblastoma cell line showed that serotonin-induced Akt phosphorylation is PI3-kinase dependent. Finally, in response to food intake, hypothalamic serotonin release was reduced in GK rats, indicating impaired responsiveness of this neurotransmitter. In conclusion, hypothalamic serotonin as insulin efficiency is impaired in diabetic GK rats. The insulin–serotonin cross-talk and impairment observed is one potential key modification in the brain during the onset of diabetes.

© 2011 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Insulin acts in the brain and more precisely at the hypothalamic level to regulate energy and glucose homeostasis (Bruning et al., 2000; Gerozissis 2008, 2010; Plum et al., 2005). An efficient insulin action in the brain is a pivotal element in maintaining numerous physiological functions. Thus, the combination of defective pancreatic beta-cell function associated to impaired insulin signaling at the peripheral and central levels contribute to the onset of diabetes (Accili, 2004; Farese et al., 2005; Lin et al.; 2004; Standaert et al., 2004).

The action of insulin in the brain is modulated by several hormones and neurotransmitters of particular interest among those factors is the neurotransmitter serotonin (5-HT) that controls food intake and energy homeostasis (Leibowitz and Alexander, 1998; Wade et al., 2008) through the same type of neurons as insulin (Xu et al., 2010; Zhou et al., 2007). Furthermore, increasing evidences have shown that serotonin regulates energy and glucose

balance through 5HT-2C receptors in the brain (Xu et al., 2010). Serotonin also improves type 2 diabetes and obesity through the same receptors (Zhou et al., 2007). These receptors are expressed by POMC neurons and their activation promotes insulin sensitivity in liver. POMC hypothalamic neurons also express insulin receptor. However, the cross-talk between serotonin and insulin signaling pathways at the hypothalamic level has received little attention specifically in type 2 diabetes models. Previous own studies have brought evidence that insulin administration in the median hypothalamus increases hypothalamic serotonin release, whereas central stimulation of the serotonergic system with dexfenfluramine, a reuptake inhibitor and enhancer of serotonin release in synapses, increases the extracellular hypothalamic insulin concentration (Orosco and Gerozissis, 2001; Orosco et al., 2000). Thus, it is plausible to hypothesize that diminished efficiency or responsiveness of the serotonergic system in the brain may promote the onset of insulin-resistance and the progress of diabetes.

To address the issue of a potential link between serotonin and brain insulin signaling in diabetes, we performed *in vivo* studies in the Goto-Kakizaki (GK) rat, a non-obese spontaneous type 2 diabetic model. This polygenic model of diabetes, produced by selective inbreeding of Wistar rats expressing glucose intolerance, presents the main features of the metabolic and hormonal disorders usually described in diabetic patients (Movassat et al., 2008;

* Corresponding author at: Laboratoire de Neuroendocrinologie Moléculaire de la Prise Alimentaire, (CNPS), UMR 8195 CNRS Université Paris Sud IBAIC, Bât. 447, 91405 Orsay cedex, France Tel.: +33 1 69 15 70 08; fax: +33 1 69 15 70 74.
    E-mail address: mohammed.taouis@u-psud.fr (M. Taouis).
  [1] These authors contributed equally.

Portha, 2005; Yamashita et al., 1996). In addition to peripheral deviations, GK rats, similarly to other type 2 diabetes models, present a number of brain dysfunctions such as leptin resistance or modification of neuropeptide Y (NPY) production, interfering with the action of insulin in energy and glucose homeostasis (Gyte et al., 2007; Maekawa et al., 2006).

In the present paper we showed that GK rats exhibited a lower insulin receptor expression levels in both hypothalamus and liver. In addition, the hypothalamic insulin-induced Akt phosphorylation, a major component of insulin signaling, is completely abolished in GK rats as compared to Wistar rats. Interestingly, we found that dexfenfluramine acute treatment induced Akt phosphorylation in the liver and hypothalamus of control but not in GK rats, linking then the inefficiency of insulin to that of dexfenfluramine. The impact of insulin-resistance on serotonin-dependent phosphorylation of Akt was confirmed in human neuroblastoma cells.

Finally, to analyze whether the serotonergic responsiveness was affected in GK rats, we investigated the hypothalamic serotonin release in response to food intake, by means of microdialysis studies. Significant differences in food intake-induced serotonin release between GK and Wistar rats were observed.

2. Research design and methods

2.1. Animals

Rat studies were carried out in agreement with the French legislation on animal experimentation and with the authorization of the French Ministry of Agriculture (Animal Health and Protection Directorate, authorization number: 91–519). Animals were maintained on a 12 h light-dark cycle. Adult male Wistar and GK rats (issued from the Paris colony, B2PE, BFA University Paris 7) aged between 8 and 12 weeks, fed standard laboratory chow (113, UAR, Epinay sur Orge, France) developed under the same conditions, were used. Food and water were available *ad libitum*.

2.2. Chemicals

Cell culture reagents from Invitrogen (Cergy-Pontoise, France), human insulin solution, serotonin creatinine sulfate complex and Wortmannin from Sigma–Aldrich (France). Antibodies directed toward Akt and phospho-(Ser 473)-Akt antibodies were from Cell Signaling (Ozyme, St Quentin en Yvelines, France). All other chemicals are from Sigma (St-Louis, USA).

2.3. Intraperitoneal insulin or dexfenfluramine injection

To test the impact of insulin or endogenous serotonin on insulin signaling, overnight fasted rats received, 30 min prior to euthanasia, an intraperitoneal (ip) injection either of saline, human insulin (1 U/kg body weight, Actrapid, Novo Nordisk, Denmark) or dexfenfluramine (5 mg/kg, Sigma–Aldrich, USA). The chosen doses of insulin and dexfenfluramine were based on previous own studies demonstrating their efficiency (Benomar et al., 2005b; Gerozissis, 2007).

2.4. Determination of plasma glucose, insulin and leptin levels

At the end of the experiment, the animals were killed by decapitation and trunk blood was collected in heparinized tubes on ice. Glucose levels were measured immediately with a blood monitoring system (Accu-Chek, Roche). The plasma was isolated and stored at −20 °C.

Table 1
Body weight, food intake, visceral adipose tissue, blood glucose, plasma hormones and basal hypothalamic serotonin (5-HT) release.

	Wistar	GK
Body weight (g)	335 ± 22.3	234 ± 4.4***
Food consumption (g/24 h)	25.0 ± 1.1	18.5 ± 1.6**
Relative food consumption (g/24 h/kg)	74.6 ± 3.3	79.1 ± 6.8
Visceral adipose tissue (g)	4.0 ± 0.6	5.4 ± 0.6**
Glucose levels (mmol/l)	4.8 ± 0.3	6.3 ± 0.8***
Insulin levels (μU/ml)	23.3 ± 1.2	41.9 ± 3.6***
Leptin levels (ng/ml)	1.5 ± 0.37	2.3 ± 0.5***
Basal 5-HT levels (pg/20 ml)	2.4 ± 0.1	2.3 ± 0.4

Data are expressed as mean ± SEM. **, ***indicate $p < 0.01$ and $p < 0.001$ respectively, when GK rats were compared to Wistar rats.
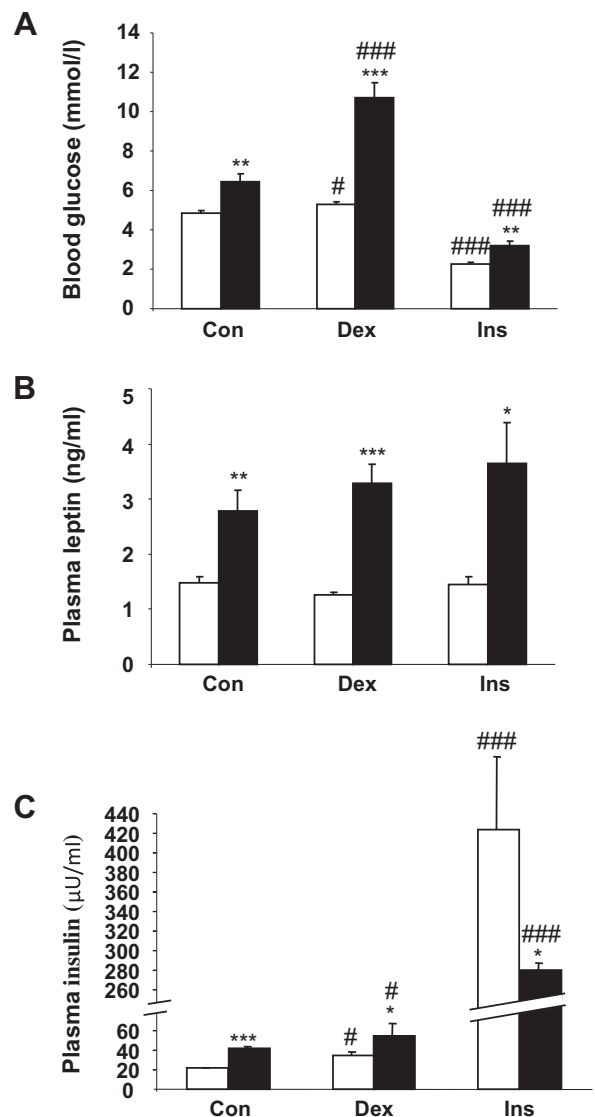$n = 6 − 8$ (for 5-HT levels, $n = 4 − 6$).

Fig. 1. Effect of dexfenfluramine or insulin injection on plasma glucose, insulin and leptin levels. Wistar (W) rats, white bars and Goto-Kakizaki (GK) rats, black bars, were injected ip 30 min before euthanasia with either saline (Con), dexfenfluramine (Dex, 5 mg/kg body weight) or human insulin (Ins, 1 U/kg body weight). Blood glucose levels (A) were immediately measured using a blood monitoring system (Accu-Chek, Roche) and plasma leptin (B) and insulin (C) levels were determined by RIA. The results are presented as means ± SEM ($n = 4 − 5$). *, **, *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, when GK rats were compared to W rats.#, ### indicate $p < 0.05$ and $p < 0.001$ when dexfenfluramine or insulin injected animals were compared to saline injected rats of the same group.

Insulin was measured by a sensitive two-step RIA (3 h preincubation, in the absence of the tracer, followed by 1 h and 30 min of incubation with tracer), using commercially available reagents (DiaSorin, Sallugia, Italy). Assay sensitivity was 2.3 ± 0.1 and 6.3 ± 0.5 µU/ml at 80% and 50%, respectively; coefficients of variation within and between assays were 5–10% and 6–10%, respectively; non-specific binding, defined as the proportion of tracer bound in the absence of antibody, was <6%. Leptin was determined by a two-step RIA, using Linco's rat leptin [125I] assay system (Linco Research, St. Charles, MO, USA). Assay sensitivity was 0.44 ± 0.01 and 1.32 ± 0.04 ng/ml at 80% and 50% respectively; coefficients of variation within and between assays 2–4.6% and 3.0–5.7%, respectively; non-specific binding was <2% (Banas et al., 2009).

2.5. Tissue sampling and Western blot analyses

Following euthanasia, hypothalami and livers were removed, immediately frozen in liquid nitrogen and stored at −80 °C. Frozen tissues were homogenized in the solubilization buffer containing 20 mM Tris–HCl (pH 7.5), 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% nonidet-P40, 10% glycerol, proteases inhibitors (0.35 mg/ml PMSF, 2 µg/ml leupeptin, 2 µg/ml aprotinin) and phosphatases inhibitors (10 mM sodium fluoride, 1 mM sodium orthovanadate, 20 mM sodium β-glycerophosphate, 10 mM benzamidine). Tissues were broken in a lysis (homogenization) apparatus (Precellys 24, Bertin technology, France). After agitation for 90 min at 4 °C, insoluble material was removed by centrifugation at 14 000 rpm, for 60 min at 4 °C. Protein concentrations of the resulting supernatant were determined using a protein assay kit (BCA) (Pierce, Perbio Science, France). Proteins, 70 µg/sample, were resolved by SDS-PAGE electrophoresis and electrotransfered to a nitrocellulose membrane. Immunoblots were blocked with 3% Bovine Serum Albumin and they were then incubated with the appropriate primary and secondary antibodies. Targeted proteins were visualized by enhanced chemiluminescence reagents (ECL detection kit, Amersham Biosciences) followed by autoradiography. Bands were quantified by densitometry, using Bio-1D software (Vilber Lourmat, France).

2.6. Surgery

Each animal was anesthetized with pentobarbital (50 mg/kg, Sanofi-Aventis, Libourne, France), pre-treated with the muscle relaxant xylasine (Rompun, Bayer Puteaux, France) and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA). A guide cannula (CMA, Carnegie Medicin AB, Stockholm, Sweden) was aimed at the space lining the paraventricular (PVN) and ventromedian (VMH) nuclei (coordinates: −1.9 mm anterior, 0.5 mm lateral, and 7 mm ventral to bregma). The dialysis probe protruded 2 mm beyond the guide tube, the tip reaching a point 9 mm ventral to dura. The guide was fixed to the skull with stainless steel screws and dental cement. One week was allowed for postoperative recovery from brain surgery, necessary for a normal sleep, feeding, and body weight gain patterns (Banas et al., 2009; Gerozissis et al., 2001). All experiments were performed in the animals' own home cages.

2.7. Microdialysis procedure

The microdialysis membranes (CMA) were 2 mm long, with a diameter of 0.5 mm and a 20 kD cut off. According to our *in vitro* calibration test, the relative recovery for serotonin was around 10% (Banas et al, 2009.). An artificial cerebrospinal fluid (Ringer type solution containing 147 mM Na^+, 2.3 mM Ca^{2+}, 4 mM K^+, and 155.6 mM Cl^-) was infused at a flow rate of 2 µl/min. Thirty microliters samples were collected every 15 min.

Brain microdialysis was performed in awake Wistar and GK rats, one week after stereotaxic implantation of the guide cannula. The animals, deprived of food at 06.00 pm the day before the experiment, had *ad libitum* access to water. The probe was inserted through the guide the next day at 09.00 am. To achieve stable serotonin levels, samples were collected following an acclimation
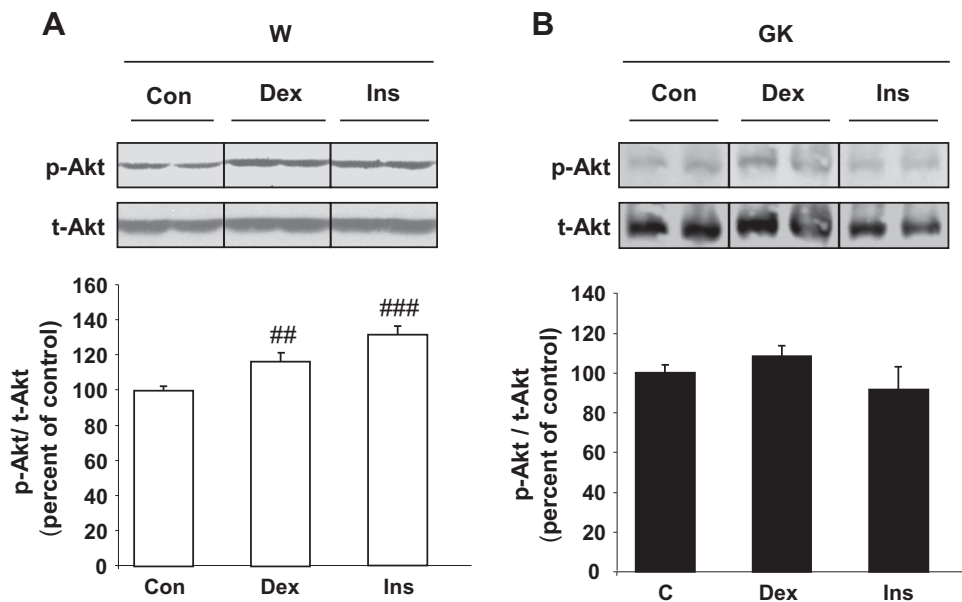

Fig. 2. Effect of dexfenfluramine or insulin injection on Akt phosphorylation in the hypothalamus. Wistar (W) rats, white bars (A) and Goto-Kakizaki (GK) rats, black bars (B), were injected ip, 30 minutes before euthanasia with either saline (Con), dexfenfluramine (Dex, 5 mg/kg body weight) or human insulin (Ins, 1 U/kg body weight). Hypothalamus lysates were subjected to Western blot analysis. Membranes were probed sequentially with anti-phospho-Akt (p-Akt) followed by anti-Akt (t-Akt) antibodies. All blots were revealed by ECL® and bands quantified by BIO1D software (Molecular Imaging, Vilber Lourma, France). The blots are representative of mean results presented in the histogram. The figure contains grouped images from representative parts of the same gel. The results are expressed as the ratio of p-Akt/t-Akt and presented as means ± SEM ($n = 6 - 10$). ##, ### indicate $p < 0.01$ and $p < 0.001$ when dexfenfluramine or insulin injected animals were compared to respective saline injected rats.
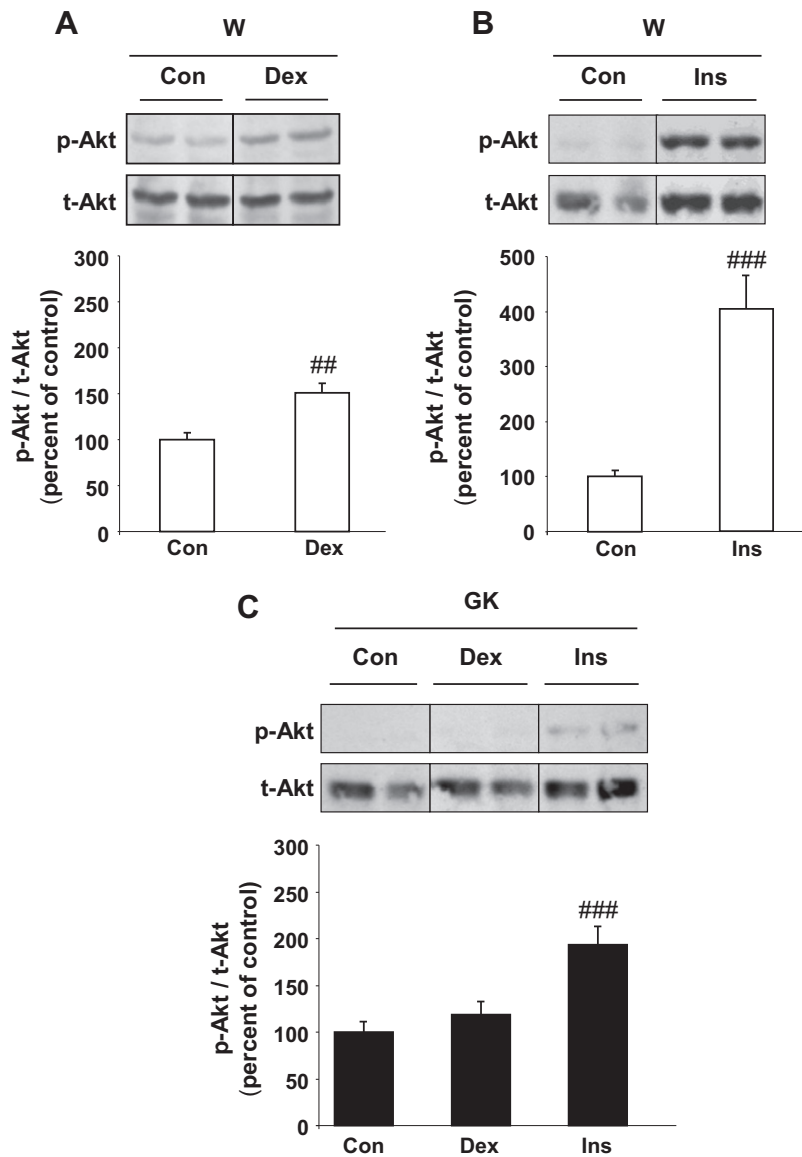
Fig. 3. Effect of dexfenfluramine or insulin injection on Akt phosphorylation in the liver. Wistar (W) rats, white bars (A,B) and Goto-Kakizaki (GK) rats, black bars (C), were injected ip 30 minutes before euthanasia with either saline (Con), dexfenfluramine (Dex, 5 mg/kg body weight) or human insulin (Ins, 1 U/kg body weight). Liver lysates were subjected to Western blot analysis. Membranes were probed sequentially with anti-phospho-Akt (p-Akt) followed by anti-Akt (t-Akt) antibodies. All blots were revealed by ECL® and bands quantified by BIO1D software (Molecular Imaging, Vilber Lourma, France). The blots are representative of mean results presented in the histogram. The figure contains grouped images from representative parts of the same gel. The results are expressed as the ratio of p-Akt/t-Akt and presented as means ± SEM ($n = 7 - 11$). ##, ### indicate $p < 0.01$ and $p < 0.001$ when dexfenfluramine or insulin injected rats were compared to respective saline injected rats.

period of 4 h after insertion of the probe (Banas et al., 2009; Gerozissis et al., 2001). After collection of four 15 min baseline samples, rats had access to *ad libitum* food intake between 03.00 and 03.30 pm. Two samples were collected during meal time and two more samples after the end of the meal.

2.8. Analysis of hypothalamic serotonin

The dialysates were analyzed by means of reverse-phase liquid chromatography (HPLC) with an electrochemical detection (Decade, Antec) at a potential of 750 mV. The chromatographic system consisted of a 20 µl sample loop leading to a 10 cm column (Colochrom, 3.2 mm internal diameter, 3 µ C-18 packing). The mobile phase consisted of an acetate buffer containing 100 µM EDTA, 1 mM octanesulfonic acid, and 6% v/v acetonitrile at pH 3.1 (Banas et al., 2009).

2.9. Histology

Rats received a lethal dose of pentobarbital, brains were removed, hardened in 10% formalin and sectioned. Probe track was identified at low magnification. Only data from animals with correct probe placements were included in data analysis.

2.10. SH-SY5Y cells stimulations

The human neuroblastoma cell line (SH-SY5Y) was obtained from Dr. B. Dufy (UMR 5543, CNRS, Bordeaux-II University, Bordeaux, France). Cells were differentiated for 15 days with retinoic acid as previously described (Benomar et al., 2005a). Serum-starved cells were incubated in serum-free DMEM in absence or presence of insulin (100 nM), serotonin (10 µM) or insulin and serotonin, for 10 min at 37 °C. To mimic insulin resistant state,

serum-starved cells were pre-incubated with insulin (100 nM) in serum-free DMEM, for 16 h and then stimulated with insulin and/ or serotonin as mentioned above. To inhibit PI3K, serum starved cells were incubated in serum-free DMEM medium in the presence of Wortmannin (100 nM), a PI3K inhibitor (Yano et al., 1993), for 30 min at 37 °C and then stimulated with insulin and/ or serotonin as mentioned above. After treatment, cells were solubilized and lysates were subjected to Western blot using adequate antibodies as above.

2.11. Statistics

Multilevel analysis of variance (ANOVA, Statistica Software) was applied for the comparisons between different treatments, two way analysis of variance (ANOVA, Statistica Software) with phenotype and treatment as the between-subjects factors was applied for the comparisons between Wistar and GK rats, received either a saline (Con), dexfenfluramine (Dex) or insulin (Ins) injection. Values are expressed as means ± SEM. For microdialysis studies, the mean 5-HT basal levels (before the meal) were calculated for each animal and the percentage of variation relative to the mean of the four baseline samples was calculated. All results were expressed as means of percentage variations ± SEM. The statistical significance for microdialysis was calculated by a one way and a two way analysis of variance followed by a Fisher PLSD test to assess the significance at each time point. Significance was set at $p < 0.05$.

3. Results

3.1. Body weight, food intake, visceral fat tissue, blood glucose, hormonal concentrations and basal hypothalamic serotonin release

Compared to age matched Wistar, body weight and food intake were significantly lower in GK rats (Table 1). In contrast, visceral adipose tissue weight was significantly higher in GK as compared to Wistar rats (Table 1). Furthermore, glycemia, insulinemia and leptinemia were also significantly increased in GK rats as compared to control rats (Table 1). Brain basal serotonin release, determined following microdialysis as described in Section 2, was similar between the two groups (Table 1).

3.2. Effect of dexfenfluramine or insulin acute treatment on glucose, insulin and leptin plasma levels

A significant difference for phenotype and treatment was observed in blood glucose concentrations, $[F(2, 24) = 21.59; p < 0.001]$, between groups and between treatments. As expected, in saline treated rats, GK group showed a significantly higher glycemia than control group (Fig. 1A). An insulin ip injection significantly lowered glycemia in Wistar and GK rats. In opposition to insulin, dexfenfluramine significantly increased glycemia in GK group and to a lesser extend in control group (Fig. 1A).

Two way analysis of variance shows a significant difference for phenotype ($p < 0.001$), but not between treatment on plasma leptin levels. Thus, independently of treatment, GK rats exhibited significant increase in plasma leptin levels as compared to Wistar rats.

Insulin ip injection largely increased plasma insulin levels in the two models by 23-fold and 8-fold in Wistar and GK rats, respectively (Fig. 1C). A significant difference for phenotype and treatment was observed $[F(1, 14) = 1.60; p < 0.001]$. Dexfenfluramine treatment significantly increased insulinemia in Wistar and GK rats (Fig. 1C) as compared to saline treatment but this effect is significantly higher in GK rats.

3.3. Effect of dexfenfluramine or insulin injection on Akt phosphorylation in hypothalamus

To determine Wistar and GK rats' insulin signaling pathway responsiveness to insulin or dexfenfluramine treatment, Akt phosphorylation was determined by Western blot analysis after normalization with total Akt. In Wistar rats, both dexfenfluramine and insulin significantly increased Akt phosphorylation as compared to saline treated rats (Fig. 2A). However, these effects were completely abolished in GK rats (Fig. 2B).

3.4. Effect of dexfenfluramine or insulin injection on Akt phosphorylation in liver

In liver of Wistar rats, dexfenfluramine (Fig. 3A) and insulin (Fig. 3B) induced a significant increase in Akt phosphorylation. Dexfenfluramine did not significantly affect Akt phosphorylation in the liver of GK (Fig. 3C). On the contrary, insulin induced Akt phosphorylation in GK rats (Fig. 3C). Notably, the amplitude of insulin-induced Akt phosphorylation was 4-fold in Wistar and 1.9-fold in GK rats.

3.5. Comparison of insulin receptor protein expression in the two models

To understand the altered efficiency of insulin on Akt phosphorylation in GK rats, we compared insulin receptor (IR) protein expression in the two groups. IR expression was significantly decreased in GK rats as compared to Wistar rats with around 40% and 60% reduction in the hypothalamus and the liver, respectively (Fig4).

3.6. Serotonin activates Akt phosphorylation in differentiated human neuronal cell line

To determine whether serotonin has a direct effect on Akt phosphorylation in a neuronal model, a dose response (Fig. 5A) and a
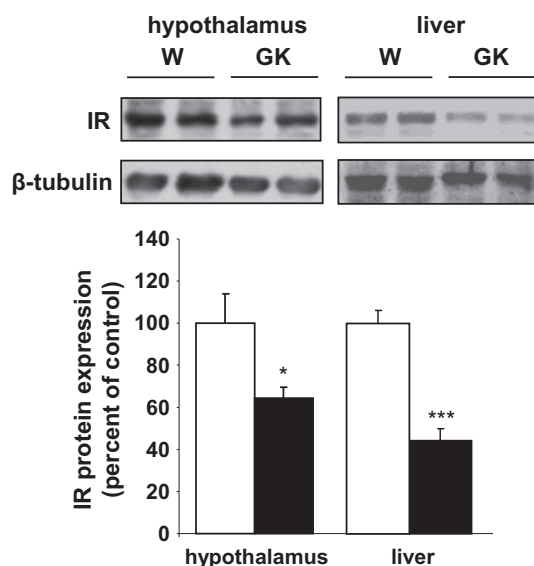

Fig. 4. Comparison of hypothalamic and hepatic IR protein expression in the two models. Hypothalamus and liver lysates from Wistar (W), white bars and Goto-Kakizaki (GK) rats, black bars, were subjected to Western blot analysis. Membranes were probed with anti-insulin receptor (IR) antibodies. The expression was normalized using β-tubulin. All blots were revealed by ECL® and bands quantified by BIO1D software (Molecular Imaging, Vilber Lourma, France). The blots are representative of mean results presented in the histogram. The results are expressed as the ratio of IR/β-tubulin and presented as means ± SEM ($n = 5 - 9$). *, *** indicate $p < 0.05$, $p < 0.001$, respectively, when GK rats were compared to W rats.
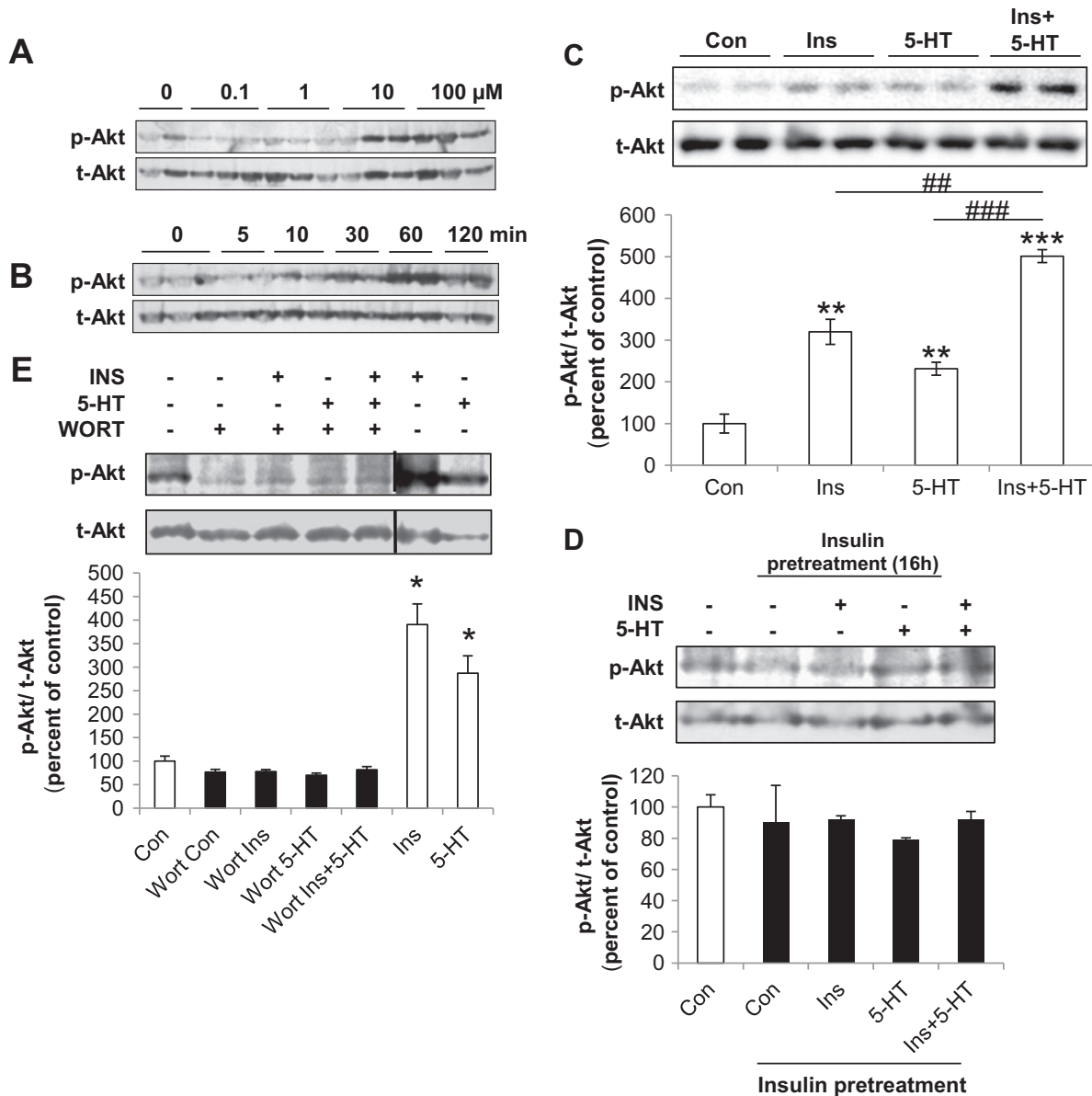
Fig. 5. Effect of serotonin on Akt phosphorylation in differentiated neuronal cells in culture. Differentiated SH-SY5Y cells were stimulated with serotonin in different time (A) and concentration (B) conditions. Differentiated SH-SY5Y cells were stimulated with insulin (100 nM), serotonin (5-HT, 10 μM) or insulin + serotonin for 10 min (C). The blots (in C) are representative of mean results presented in the histogram. The results are expressed as the ratio of p-Akt/t-Akt and presented as means ± SEM ($n = 3$). **, indicate $p < 0.01$ and $p < 0.001$, when insulin, serotonin or insulin + serotonin stimulated cells were compared to controls (Con). #, ## indicate $p < 0.01$ and $p < 0.001$ when insulin and serotonin alone were compared with insulin + serotonin stimulated cells. (D) Differentiated cells without pretreatment, white bars, or pretreated with insulin (100 nM) for 16 h, black bars were washed and stimulated with insulin (100 nM), serotonin (5-HT, 10 μM) or insulin + serotonin for 10 min. (E) Differentiated SH-SY5Y cells without pretreatment, white bars or treated with Wortmannin for 30 min, black bars, and were then stimulated with insulin (100 nM), serotonin (5-HT, 10 μM) or insulin + serotonin for 10 min. Cell lysates were subjected to Western blot analysis. Membranes were probed sequentially with anti-phospho-Akt (p-Akt) followed by anti-Akt (t-Akt) antibodies. All blots were revealed by ECL® and bands quantified by BIO1D software (Molecular Imaging, Vilber Lourma, France). The blots are representative of mean results presented in the histogram. Figures (E) contain grouped images from representative parts of the same gel. The results are expressed as the ratio of p-Akt/t-Akt and presented as means ± SEM ($n = 3$) where * indicates $p < 0.05$.

kinetic (Fig. 5B) experiment were performed in differentiated SH-SY5Y cells. Serotonin activates Akt phosphorylation from 10 μM (Fig. 5A) and using this concentration serotonin stimulates Akt phosphorylation since 10 min of treatment and increased until 60 min and then declines at 120 min (Fig. 5B). Based on literature (Prosser et al., 2006) and on the indications obtained above (Fig. 5A and B) for serotonin together with previous studies for insulin (Benomar et al., 2005a), cells were then treated for 10 min with 10 μM serotonin or 100 nM insulin or both. Both insulin and serotonin induced Akt phosphorylation (Fig. 5C). When insulin and serotonin were applied simultaneously, the phosphorylation of

Akt was significantly higher than that obtained by serotonin or insulin alone (Fig 5C).

3.7. Effect of serotonin on Akt phosphorylation following overexposure to insulin

To mimic insulin resistant state, differentiated SH-SY5Y cells were pretreated with 100 nM insulin for 16 hours (Benomar et al., 2005a). After several washes, cells were acutely challenged with serotonin, insulin or both insulin and serotonin. As expected, insulin pretreatment completely inhibited insulin acute effect, but
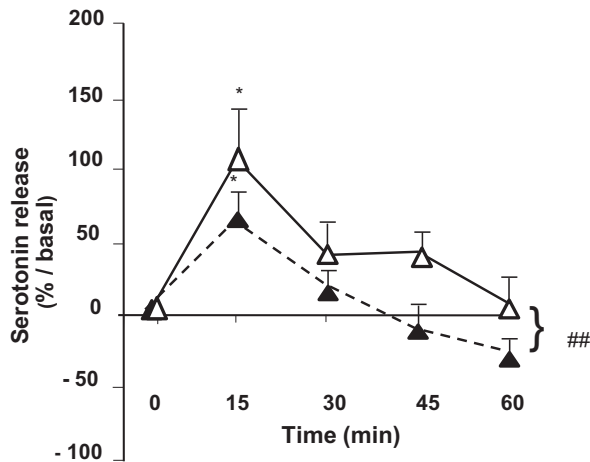
Fig. 6. Hypothalamic serotonin response to food intake. Serotonin concentrations were measured in 15 min samples of microdialysis, before (4 samples), during (2 samples) and after (2 samples) the meal presentation. White triangles represent Wistar and black triangles GK rats. The percentage of variation relative to the mean of the four baseline samples was calculated. All results were expressed as means of percentage variations ± SEM ($n = 4$). *indicates $p < 0.05$, a difference towards baseline levels (0 level). ##indicates $p < 0.01$, a difference within Wistar and Goto Kakizaki groups.

also clearly diminished serotonin effect on Akt phosphorylation (Fig 5D).

3.8. Serotonin activates Akt phosphorylation through a PI3K dependent mechanism

Since we described above that the alteration of insulin signaling impaired serotonin-dependent Akt phosphorylation we attempted to determine whether serotonin activates Akt phosphorylation through PI3K pathway. Thus, cells were pretreated with 100 nM Wortmannin (inhibitor of PI3K) 30 minutes prior to treatment with serotonin, insulin or both insulin and serotonin. Wortmannin pre-treatment completely abolished both insulin and serotonin effect on Akt phosphorylation (Fig. 5E).

3.9. Reduced hypothalamic serotonin response to food intake in GK rats

To evaluate the responsiveness of serotonin to food intake and to determine if this response is altered in a type 2 diabetes model, extracellular hypothalamic release using microdialysis technique was measured as described in materials and methods. The meal significantly increased serotonin release in Wistar and GK rats during meal time (Fig6). However, the time course of serotonin release during and after the meal was significantly different between the two models [$F(1,40) = 20,97$; $p < 0,01$], with a significantly lower serotonin release in GK rats as compared to Wistar.

4. Discussion

There are strong evidences that inefficiency of brain insulin action may be involved in the initiation and the progress of type 2 diabetes (Accili, 2004; Gerozissis, 2010; Konner et al., 2007; Obici et al., 2002; Schwartz and Porte, 2005). To address this issue, we compared the efficiency of insulin signaling, as mirrored by Akt phosphorylation, in GK (type 2 diabetes model) and Wistar rats. This study demonstrates that insulin failed to phosphorylate Akt in the hypothalamus of the diabetic phenotype. Further, we have looked for a potential association between alteration of central

insulin and serotonin action in the diabetic model. Therefore, to stimulate the serotonergic system, we performed an acute injection of dexfenfluramine, 30 min before sacrifice, in a dose known to induce hypophagia (Gerozissis, 2007). To investigate the effect of serotonin on insulin signaling pathways we have extended our study to a human neuronal cell line.

We showed that serotonin, as insulin, activates Akt phosphorylation in the hypothalamus of Wistar rats, but not in diabetic GK rats indicating an alteration of the responsiveness and the efficiency of the serotonergic system. In non-diabetic rats, dexfenfluramine-induced Akt phosphorylation could be attributed to either a direct effect of serotonin in the hypothalamus as previously suggested (Orosco and Gerozissis, 2001; Orosco et al., 2000) or to a subsequent insulinemia increase in response to dexfenfluramine treatment. However, in GK rats even if dexfenfluramine increased insulinemia more than in Wistar rats, it did not induce hypothalamic Akt phosphorylation, suggesting an insulin resistant state and/or alteration of serotonergic efficiency (Fig. 7). Supporting this notion, the regulation of specific serotonergic receptors is altered in the brain of streptozotocin induced diabetic rats (Abraham et al., 2010).

In the liver and as expected, insulin induced a 4-fold Akt phosphorylation in Wistar rats. Similarly to the hypothalamus, dexfenfluramine induced Akt phosphorylation in the liver of Wistar rats and was ineffective in GK, favoring the hypothesis that the effect of serotonin is not associated to increased peripheral insulinemia. In opposition to serotonin, insulin was still effective in GK rats, inducing a 2-fold Akt phosphorylation above basal, indicating a partial peripheral insulin resistance.

In an attempt to check whether serotonin acts directly on Akt phosphorylation, we have used a human neuroblastoma cell line (SH-SY5Y). These cells express endogenous insulin and serotonin receptors (Benomar et al., 2005a; Schmuck et al., 1994). Here, we demonstrated that serotonin per se was able to activate the phosphorylation of Akt confirming our observations in the hypothalamus. Using a PI3K inhibitor, we show that serotonin activates Akt phosphorylation through a PI3K dependent mechanism. To mimic a dysfunction such as hyperinsulinemia, neuronal cells were overexposed to insulin during 16 h, condition that we have previously demonstrated to induce insulin resistance (Benomar et al., 2005a), and then subjected to serotonin treatment. In line with the results obtained in the hypothalamus of GK rats and reinforcing this finding, our data indicate that, in neuronal cells, serotonin is unable to phosphorylate Akt after chronic insulin pre-treatment.

To assess mechanisms involved in the altered insulin efficiency in the diabetic rat, insulin receptor expression was determined at the protein level. Compared to Wistar rats, diminished insulin receptor expression was found in the liver and the hypothalamus of GK rats. Consequently, the alterations on Akt phosphorylation of the diabetic model could be associated to lower insulin receptor expression probably due to the hyperinsulinemia of GK rats. Interestingly, the insulin receptor decrease in the diabetic model is higher in the liver (55%) than in the hypothalamus (36%). Altered insulin transport of plasma insulin in the brain, known to occur in metabolic dysfunctions (Israel et al., 1993; Reger and Craft, 2006; Schwartz and Porte, 2005), could explain the difference of the impairment between the periphery and the brain. Additional modifications of negative regulators of insulin signaling pathways or/and other factors might be involved in hypothalamic insulin responsiveness impairment (Dadke et al., 2000). Altered endogenous hypothalamic serotonergic efficiency might be one of them. Although insulin signaling was affected in both the brain and the liver of the GK rat, the sensitivity of exogenous insulin to lower basal glycemia was maintained. The gluco-regulatory efficiency of insulin in GK could depend on differential effects of insulin in organs not investigated in the present study, such as the muscle
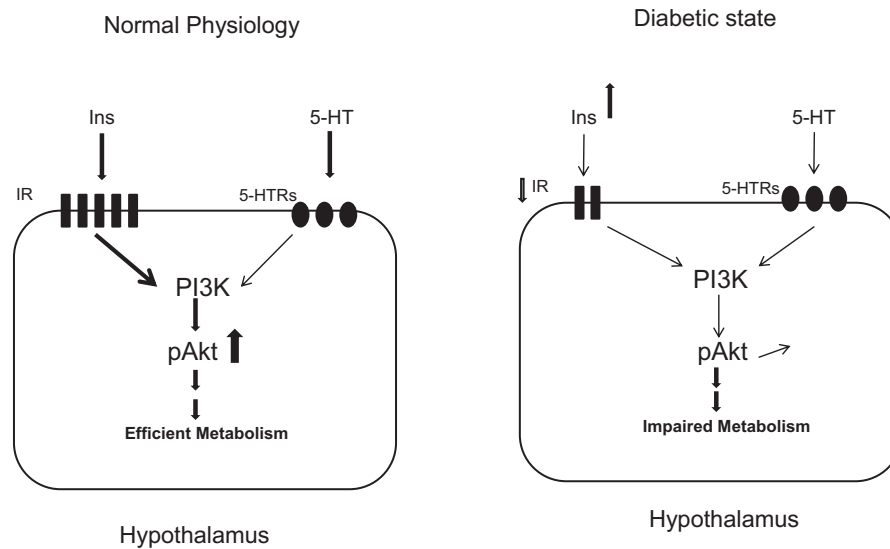
Fig. 7. A summary model. This model summarizes the serotonin–insulin cross-talk in non-diabetic and diabetic state in the hypothalamus. In normal physiology, both insulin (Ins) and serotonin (5-HT) activate PI3-kinase (PI3K)/Akt signaling pathway through their insulin receptor (IR) and serotonin receptors (5-HTRs). Food intake (FI) increased 5-HT release in the hypothalamus less in GK than in normal rats. In diabetic state, IR is down-regulated leading to reduced PI3K/Akt activation promoting then, together with altered serotonergic responsiveness, the impairment of metabolism.

(Sajan et al., 2009) and adipose tissue, or on additional signaling elements or pathways that are differentially responding in diabetes (Lin et al., 2004; Sajan et al., 2009; Standaert et al., 2004).

Peripheral insulin injection lowered circulating glucose without significant change in plasma leptin levels in the two phenotypes. Dexfenfluramine increased plasma insulin in both Wistar and GK rats. Dexfenfluramine injection increased slightly but significantly blood glucose in Wistar rats, in line with observations obtained in mice following serotonin treatment (Watanabe et al., 2010). Interestingly, dexfenfluramine dramatically increased glycaemia in GK diabetic rats, suggesting perturbation of serotonergic efficacy. The above results obtained by a single acute stimulation of the serotonergic system, differ from observations on long-term treated diabetic patients or insulin resistant rats with serotonin reuptake inhibitors shown to lower glycaemia or hepatic glucose production (Buhl et al., 2010; Proietto et al., 1994; Xu et al., 2010; Zhou et al., 2007). Furthermore, the effect of dexfenfluramine in GK rats regarding hyperglycemia could be, at least in part, attributed to a potential increase of noradrenalin since dexfenfluramine has been described as potent substrate for norepinephrine transporters (Rothman et al., 2003). Furthermore despite the alteration of the serotonergic system in GK rats, the relative food intake was not affected as compared to Wistar rats. This could be attributed to increased leptinemia and insulinemia that may still partially regulate food intake even if a relative resistance to these hormones was reported in GK rats and we cannot exclude other compensatory mechanisms not yet identified.

To explore eventual endogenous alterations of the serotonergic system in diabetes, we performed brain microdialysis in vigilant rats. Under chow diet, basal hypothalamic serotonin release before the chow meal was identical in Wistar and GK rats. Food intake increased hypothalamic serotonin release in the two groups. However, the amplitude of food-induced neurotransmitter release in the diabetic rats was significantly lower than that of healthy controls, suggesting a diminished responsiveness of the serotonergic system in the hypothalamus of GK rats, following physiological stimuli. In line with previous indirect observations in patients with metabolic syndrome (Muldoon et al., 2004), altogether the present data and previous own observations for increased insulin concentrations in the hypothalamus after stimulation of the serotonergic

system (Orosco and Gerozissis, 2001; Orosco et al., 2000) suggest that reduced brain serotonin release might have a negative impact on brain insulin efficiency.

Globally, the present study demonstrates that insulin- and serotonin-dependent Akt phosphorylation is altered in the hypothalamus and liver of diabetic GK rats and the effect of serotonin on Akt phosphorylation could be direct as evidenced in a human neuronal cell line. The impairment of hypothalamic insulin efficiency in GK rats could be attributed, at least in part, to modifications of nutrition-depended serotonin responsiveness or/and altered efficiency of the neurotransmitter (Fig. 7). Our findings on the central insulin and serotonin communication in normal function and metabolic dysfunctions are reinforced by recent loose of function and pharmacological studies (Xu et al., 2010; Zhou et al., 2007). Whether the alterations of insulin and serotonin signaling observed in the type 2 diabetes phenotype are a consequence or a starting point for the development of metabolic dysfunctions are major issues for further investigation. Nonetheless, our work reveals an unexplored aspect of the central mechanisms involved in insulin–serotonin cross-talk.

Acknowledgements

References

Abraham, P.M., Anju, T.R., Jayanarayanan, S., Paulose, C.S., 2010. Serotonergic receptor upregulation in cerebral cortex and down regulation in brainstem of streptozotocin induced diabetic rats: antagonism by pyridoxine and insulin. Neurosci. Lett. 483, 23–27.
Accili, D., 2004. Lilly lecture 2003: the struggle for mastery in insulin action: from triumvirate to republic. Diabetes 53, 1633–1642.
Banas, S.M., Rouch, C., Kassis, N., Markaki, E.M., Gerozissis, K., 2009. A dietary fat excess alters metabolic and neuroendocrine responses before the onset of metabolic diseases. Cell. Mol. Neurobiol. 29, 157–168.
Benomar, Y., Roy, A.F., Aubourg, A., Djiane, J., Taouis, M., 2005a. Cross down-regulation of leptin and insulin receptor expression and signalling in a human neuronal cell line. Biochem. J. 388, 929–939.
Benomar, Y., Wetzler, S., Larue-Achagiotis, C., Djiane, J., Tome, D., Taouis, M., 2005b. In vivo leptin infusion impairs insulin and leptin signalling in liver and hypothalamus. Mol. Cell. Endocrinol. 242, 59–66.

Bruning, J.C., Gautam, D., Burks, D.J., Gillette, J., Schubert, M., Orban, P.C., Klein, R., Krone, W., Muller-Wieland, D., Kahn, C.R., 2000. Role of brain insulin receptor in control of body weight and reproduction. Science 289, 2122–2125.

Buhl, E.S., Jensen, T.K., Jessen, N., Elfving, B., Buhl, C.S., Kristiansen, S.B., Pold, R., Solskov, L., Schmitz, O., Wegener, G., Lund, S., Petersen, K.F., 2010. Treatment with an SSRI antidepressant restores hippocampo-hypothalamic corticosteroid feedback and reverses insulin resistance in low-birth-weight rats. Am. J. Physiol. Endocrinol. Metab. 298, E920–E929.

Dadke, S.S., Li, H.C., Kusari, A.B., Begum, N., Kusari, J., 2000. Elevated expression and activity of protein-tyrosine phosphatase 1B in skeletal muscle of insulin-resistant type II diabetic Goto-Kakizaki rats. Biochem. Biophys. Res. Commun. 274, 583–589.

Farese, R.V., Sajan, M.P., Standaert, M.L., 2005. Insulin-sensitive protein kinases (atypical protein kinase C and protein kinase B/Akt): actions and defects in obesity and type II diabetes. Exp. Biol. Med. (Maywood) 230, 593–605.

Gerozissis, K., 2007. Brain insulin signaling regulation by leptin and serotonin. Potential impact on the development of metabolic diseases., Diabetologia. pp. (Suppl 1):A0015, 2007.

Gerozissis, K., 2008. Brain insulin, energy and glucose homeostasis; genes, environment and metabolic pathologies. Eur. J. Pharmacol. 585, 38–49.

Gerozissis, K., 2010. The Brain insulin connection, metabolic diseases and related pathologies. In: Scy, C. (Ed.), Diabetes, Insulin and Alzheimer's Disease, Research and Perspectives in Alzheimer's Disease. Springer, Verlag Berlin Heidelberg, pp. 21–42.

Gerozissis, K., Rouch, C., Lemierre, S., Nicolaidis, S., Orosco, M., 2001. A potential role of central insulin in learning and memory related to feeding. Cell. Mol. Neurobiol. 21, 389–401.

Gyte, A., Pritchard, L.E., Jones, H.B., Brennand, J.C., White, A., 2007. Reduced expression of the KATP channel subunit, Kir6.2, is associated with decreased expression of neuropeptide Y and agouti-related protein in the hypothalami of Zucker diabetic fatty rats. J. Neuroendocrinol 19, 941–951.

Israel, P.A., Park, C.R., Schwartz, M.W., Green, P.K., Sipols, A.J., Woods, S.C., Porte Jr., D., Figlewicz, D.P., 1993. Effect of diet-induced obesity and experimental hyperinsulinemia on insulin uptake into CSF of the rat. Brain Res. Bull. 30, 571–575.

Konner, A.C., Janoschek, R., Plum, L., Jordan, S.D., Rother, E., Ma, X., Xu, C., Enriori, P., Hampel, B., Barsh, G.S., Kahn, C.R., Cowley, M.A., Ashcroft, F.M., Bruning, J.C., 2007. Insulin action in AgRP-expressing neurons is required for suppression of hepatic glucose production. Cell Metab. 5, 438–449.

Leibowitz, S.F., Alexander, J.T., 1998. Hypothalamic serotonin in control of eating behavior, meal size, and body weight. Biol. Psychiatry 44, 851–864.

Lin, X., Taguchi, A., Park, S., Kushner, J.A., Li, F., Li, Y., White, M.F., 2004. Dysregulation of insulin receptor substrate 2 in beta cells and brain causes obesity and diabetes. J. Clin. Invest. 114, 908–916.

Maekawa, F., Fujiwara, K., Kohno, D., Kuramochi, M., Kurita, H., Yada, T., 2006. Young adult-specific hyperphagia in diabetic Goto-kakizaki rats is associated with leptin resistance and elevation of neuropeptide Y mRNA in the arcuate nucleus. J. Neuroendocrinol. 18, 748–756.

Movassat, J., Bailbe, D., Lubrano-Berthelier, C., Picarel-Blanchot, F., Bertin, E., Mourot, J., Portha, B., 2008. Follow-up of GK rats during prediabetes highlights increased insulin action and fat deposition despite low insulin secretion. Am. J. Physiol. Endocrinol. Metab. 294, E168–E175.

Muldoon, M.F., Mackey, R.H., Williams, K.V., Korytkowski, M.T., Flory, J.D., Manuck, S.B., 2004. Low central nervous system serotonergic responsivity is associated with the metabolic syndrome and physical inactivity. J. Clin. Endocrinol. Metab. 89, 266–271.

Obici, S., Zhang, B.B., Karkanias, G., Rossetti, L., 2002. Hypothalamic insulin signaling is required for inhibition of glucose production. Nat. Med. 8, 1376–1382.

Orosco, M., Gerozissis, K., 2001. Macronutrient-induced cascade of events leading to parallel changes in hypothalamic serotonin and insulin. Neurosci. Biobehav. Rev. 25, 167–174.

Orosco, M., Rouch, C., Gerozissis, K., 2000. Activation of hypothalamic insulin by serotonin is the primary event of the insulin–serotonin interaction involved in the control of feeding. Brain Res. 872, 64–70.

Plum, L., Schubert, M., Bruning, J.C., 2005. The role of insulin receptor signaling in the brain. Trends Endocrinol. Metab. 16, 59–65.

Portha, B., 2005. Programmed disorders of beta-cell development and function as one cause for type 2 diabetes? The GK rat paradigm. Diabetes Metab. Res. Rev. 21, 495–504.

Proietto, J., Thorburn, A.W., Fabris, S., Harrison, L.C., 1994. Effects of dexfenfluramine on glucose turnover in non-insulin-dependent diabetes mellitus. Diabetes Res. Clin. Pract. 23, 127–134.

Prosser, R.A., Lee, H.M., Wehner, A., 2006. Serotonergic pre-treatments block in vitro serotonergic phase shifts of the mouse suprachiasmatic nucleus circadian clock. Neuroscience 142, 547–555.

Reger, M.A., Craft, S., 2006. Intranasal insulin administration: a method for dissociating central and peripheral effects of insulin. Drugs Today (Barc) 42, 729–739.

Rothman, R.B., Clark, R.D., Partilla, J.S., Baumann, M.H., 2003. (+)-Fenfluramine and its major metabolite, (+)-norfenfluramine, are potent substrates for norepinephrine transporters. J. Pharmacol. Exp. Ther. 305, 1191–1199.

Sajan, M.P., Standaert, M.L., Rivas, J., Miura, A., Kanoh, Y., Soto, J., Taniguchi, C.M., Kahn, C.R., Farese, R.V., 2009. Role of atypical protein kinase C in activation of sterol regulatory element binding protein-1c and nuclear factor kappa B (NFkappaB) in liver of rodents used as a model of diabetes, and relationships to hyperlipidaemia and insulin resistance. Diabetologia 52, 1197–1207.

Schmuck, K., Ullmer, C., Engels, P., Lubbert, H., 1994. Cloning and functional characterization of the human 5-HT2B serotonin receptor. FEBS Lett. 342, 85–90.

Schwartz, M.W., Porte Jr., D., 2005. Diabetes, obesity, and the brain. Science 307, 375–379.

Standaert, M.L., Sajan, M.P., Miura, A., Kanoh, Y., Chen, H.C., Farese Jr., R.V., Farese, R.V., 2004. Insulin-induced activation of atypical protein kinase C, but not protein kinase B, is maintained in diabetic (ob/ob and Goto-Kakazaki) liver. Contrasting insulin signaling patterns in liver versus muscle define phenotypes of type 2 diabetic and high fat-induced insulin-resistant states. J. Biol. Chem. 279, 24929–24934.

Wade, J.M., Juneja, P., MacKay, A.W., Graham, J., Havel, P.J., Tecott, L.H., Goulding, E.H., 2008. Synergistic impairment of glucose homeostasis in ob/ob mice lacking functional serotonin 2C receptors. Endocrinology 149, 955–961.

Watanabe, H., Akasaka, D., Ogasawara, H., Sato, K., Miyake, M., Saito, K., Takahashi, Y., Kanaya, T., Takakura, I., Hondo, T., Chao, G., Rose, M.T., Ohwada, S., Watanabe, K., Yamaguchi, T., Aso, H., 2010. Peripheral serotonin enhances lipid metabolism by accelerating bile acid turnover. Endocrinology 151, 4776–4786.

Xu, Y., Berglund, E.D., Sohn, J.W., Holland, W.L., Chuang, J.C., Fukuda, M., Rossi, J., Williams, K.W., Jones, J.E., Zigman, J.M., Lowell, B.B., Scherer, P.E., Elmquist, J.K., 2010. 5-HT2CRs expressed by pro-opiomelanocortin neurons regulate insulin sensitivity in liver. Nat. Neurosci. 13, 1457–1459.

Yamashita, S., Nakamura, T., Shimomura, I., Nishida, M., Yoshida, S., Kotani, K., Kameda-Takemuara, K., Tokunaga, K., Matsuzawa, Y., 1996. Insulin resistance and body fat distribution. Diabetes Care 19, 287–291.

Yano, H., Nakanishi, S., Kimura, K., Hanai, N., Saitoh, Y., Fukui, Y., Nonomura, Y., Matsuda, Y., 1993. Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. J. Biol. Chem. 268, 25846–25856.

Zhou, L., Sutton, G.M., Rochford, J.J., Semple, R.K., Lam, D.D., Oksanen, L.J., Thornton-Jones, Z.D., Clifton, P.G., Yueh, C.Y., Evans, M.L., McCrimmon, R.J., Elmquist, J.K., Butler, A.A., Heisler, L.K., 2007. Serotonin 2C receptor agonists improve type 2 diabetes via melanocortin-4 receptor signaling pathways. Cell. Metab. 6, 398–405.

RESULTS

ARTICLE 2

Title

High fat diet induces a reversible depressive-like behavior in rats, associated with a down-regulation of the PI3K/Akt/GSK3β pathway in the dentate gyrus.

Ioannis Papazoglou[1,2], Arnaud Jean[1,2], Alain Aubourg[1,2], Arieh Gertler[3], Mohammed Taouis[1,2], Claire-Marie Vacher[1,2*]

[1] Neuroendocrinologie Moléculaire de la Prise Alimentaire, University of Paris-Sud 11, UMR 8195, Orsay, F-91405, France.

[2] Neuroendocrinologie Moléculaire de la Prise Alimentaire, CNRS, Centre de Neurosciences, Paris-Sud UMR8195, Orsay, F-91405, France.

[3] The Hebrew University of Jerusalem, Faculty of Agricultural, Food and Environmental Quality Sciences Institute of Biochemistry Food Science and Nutrition, Rehovot 76100 Israel

* Corresponding author

Abstract

Accumulating studies show that the metabolic syndrome, including obesity and type 2 diabetes, increases the risk of mood disorders such as depression. Nevertheless, the mechanisms underlying this association are not clearly understood. In the current study, we hypothesized that leptin- and insulin-resistance impair the activation of serotonin signaling in the dentate gyrus (DG). To address, this question, we analyzed the behavioral and brain-signaling consequences of a high fat (HF) diet on adult Wistar rats. We first showed that 8-9-week long HF diet induced significant overweight and hyperglycemia as well as depressive-like behavior compared with control chow diet, as evidenced in saccharin preference and swim forced tests. By using an *ex vivo* approach on brain slices, we demonstrated that leptin, insulin and serotonin activate the PI3K/Akt/GSK3β signaling pathway in the DG and that a 16-week HF-diet feeding results in a decrease of this activation. Furthermore, our data demonstrated a significantly negative correlation between depressive-like behavior and serotonin-induced phosphorylation of GSK3β in the subgranular cells of the DG. Interestingly, a standard food substitution for 6 extra-weeks induced a total loss of depressive symptoms, whereas physiological parameters (body weight and glycemia) remained significantly higher than normal. In addition, food restoration reversed the activation of the PI3K/Akt/GSK3β signaling pathway by leptin, insulin and serotonin in the DG. In conclusion, our data by providing evidence for the existence of a cross-talk between leptin, insulin and serotonin signaling in the brain, suggest novel molecular mechanisms linking metabolic syndrome and depression, and open new avenues in the understanding and management of these pathologies.

Introduction

Obesity and type 2 diabetes represent two of the most rapidly increasing worldwide epidemics[1, 2]. These two pathologies, usually associated to the metabolic syndrome, correlate in both symptoms and mechanistic patterns[3, 4], and raise the risk of developing depression[5, 6], the third most frequent mental disorder in Europe[7]. Reciprocally, depressive symptoms have been identified as risk factors for the development of type 2 diabetes and/or obesity[8-10]. More globally, a recent large-scale meta-analysis of earlier studies has confirmed the two-way relationship between metabolic syndrome and depression [11].

Metabolic syndrome includes a resistance to insulin and leptin [4], two hormones produced by pancreatic beta cells and white adipocytes, respectively, which play a crucial role in the regulation of energy homeostasis notably by modulating the activity of various specialized neuronal circuits in the central nervous system[12]. These hormones activate common signaling pathways[4] and as a result they can alter the neuronal response of each other in vitro[13] and in vivo[14]. This cross-talk involves the insulin receptor substrate/phosphatidylinositol-3 kinase/Akt (IRS/PI3K/Akt) signaling pathway, which is essential for the initiation of the majority of neuronal functions induced by insulin[15] and leptin[16], and may contribute to the reciprocal link existing between type 2 diabetes and obesity.

The neurotransmitter serotonin (5-HT) is a major key player in the regulation of mood and the impairment of its production, reuptake and action contributes essentially to depressive syndromes[17]. Importantly, one of the initial actions of 5-HT mediating its mood enhancing properties is the inhibition of glycogen synthase kinase 3β (GSK3β) by phosphorylation on Ser9 residue [18-20], an important downstream target of the PI3K/Akt pathway[21]. Analogous properties in blocking the activity of GSK3β are exhibited by insulin[22] and leptin[23], which assign them as candidates for direct anti-depressive action and interaction with the serotonergic signal transduction.

It has been suggested that the association between depression and obesity may be due to impaired leptin activity in the hippocampus, a brain region involved in mood disorders[24]. In addition, putative biochemical and cellular mechanisms have been proposed by studies that describe how the dietary[24-26] (e.g. high fat diet) or the genetic (e.g. under-expression of the insulin receptor[27] or leptin receptor[28]) manipulation, which decrease the sensitivity and efficiency of these hormones, can further induce or exacerbate depressive-like symptoms in rodents. However, the precise mechanisms underlying this link remained unknown. Considering that we have previously demonstrated that insulin and 5-HT signaling pathways can interact via PI3K/Akt in neurons[29], we hypothesized that insulin and leptin resistance may affect 5-HT signaling in the dentate gyrus of the hippocampus, and in particular the subgranular layer. Indeed, this area highly expresses the receptors of insulin [30] and leptin [31, 32] and their substrates [30, 33], and is considered as an important target of antidepressant action, notably due to its intense serotonergic innervations [34, 35].

The aim of this study was thus to gain evidence about how leptin- and insulin-resistance contribute to a depressive like behavior by interacting with the serotonergic system. To address this question, we submitted rats to a high fat (HF) diet and we studied the correlations between diet-induced obesity, type 2 diabetes, depression, and the sensitivity to leptin, insulin and 5-HT in subgranular neurons. Here we validated that rats fed a high fat diet (HF) exhibit a reversible higher risk of developing depressive syndrome, as attested by behavioral tests such as the forced swimming test (FST) and the sucrose preference test (SPT). Furthermore, we demonstrated that this may be due, at least in part, to a cross-desensitization of the subgranular neurons to leptin, insulin and 5-HT at the level of the PI3K/Akt/GSK3β signaling pathway.

Materials and Methods

Animals and diets

Eight weeks old male Wistar rats (RjHan:WI) were obtained from Janvier (Saint-Berthevin, France). The rats were pair-housed and kept under a 12-h light/dark cycle in a temperature-controlled environment at 22 °C. After one week of acclimatization, the animals received *ad libitum* experimental diets: either standard chows (#113 Safe, Augy, France) (C group) or a purified high fat diet (#235 version 2 Safe) (HF group). In the HF diet, 46% of the energy content derived from lipids versus 17% in the standard diet. Furthermore, the respective caloric intake differed between both diets: 4397 kcal/kg in the HF diet versus 3 000 kcal/kg in the standard one. After 10 weeks of experimental diets, half of the HF group was subjected to a restoration of the standard diet for 6 extra-weeks (HF/C group), whereas the second half of this group was kept under a HF diet (Fig. 1). Body weight and food intake were recorded throughout the experiments. Rats were always killed at the same time of the day (4-5 h after lights on). Experiments were performed according to European legal requirements (Decree 86/609/EEC).

Brain slice experiments

Ex vivo experiments were modified from previous studies[36, 37]. Rats fed a control or a HF diet were decapitated under isofluorane anaesthesia, and their brain immediately removed and transferred into ice-cold oxygenated Krebs-Henseleit bicarbonate buffer (K3753, Sigma, Lyon, France) supplemented with 24.9 mM $NaHCO_3$, 2 mM $CaCl_2$, 11 mM ascorbic acid and 315 mM thiourea (Sigma). Three hundred μm-thick slices of hippocampus were prepared caudally from Bregma -1.8 mm to -4.8 mm with the help of a stereotaxic atlas (Paxinos and Watson, 5th edition). Slices were halved with a razor blade through the medial line and left for a 1 h recovery at RT in oxygenated buffer. Hemislices were then transferred and immersed in

the chamber system (Campden Instruments Ltd, Leicester, UK) continuously oxygenated and perfused with Krebs buffer with a flow rate of 2 mL/min, and slowly warmed to 35 °C. Slices were incubated for 5 min with 5-HT (10 µM; Sigma), insulin (100 nM; Actrapid®, Novo Nordisk, La Défense, France), or leptin (10 nM; Protein Laboratories Rehovot, Rehovot, Israel). Control slices were incubated with buffer alone.

Immunohistochemistry

Tissue preparation. After the 5-min stimulation in the perfusion system, slices were directly fixed by immersion in cold 4% paraformaldehyde in PBS for 2 hours before being cryoprotected in 20% sucrose for 2 hours at 4 °C. They were then frozen and cut coronally with a cryostat at -16°C into 20 µm thick sections. The sections were thawed onto Superfrost Plus slides (Thermo Scientific) so that all the conditions (control and treated) were present on the same slide, and finally stored at -20 °C until use.

Immunofluorescence. Multiple immunofluorescent stainings including the detection of pAkt, pGSK3β, Ki67, Hu protein and calretinin were performed on brain sections. The main lines of the immunohistochemical protocol were the same as those previously described with slight adaptations[38]. Briefly, hippocampal sections were incubated overnight at 4 °C with a combination of rabbit anti-pAkt (Ser473) (D9E) XP™ (1:100; Cell Signaling Technology, Beverly, MA, USA), rabbit anti-pGSK3β (Ser9) (D85E12) XP® (1:100; Cell Signaling Technology), rabbit monoclonal anti-Ki67 (1:100, Novus Biologicals, Cambridge, UK) and mouse anti-HuC/D (1:50, Molecular Probes, Life Technologies, Saint-Aubin, France). Primary antibodies were visualized by incubating the sections for 1 h at RT with Alexa-488, Alexa-546 or Alexa 633 (Life Technologies) conjugated donkey anti-goat, rabbit, or mouse antibodies (1:400). Sections were finally counterstained with DAPI.

Confocal microscopy and quantification. Immunofluorescence was examined by confocal microscopy (Zeiss LSM 700 system; Zeiss microscopy, Jena, Germany). Series of optical sections were collected through the Z axis at 1 µm intervals and averaged four times. Four diode lasers at 405 nm, 488 nm, 555 nm and 639 nm were used for the excitation of DAPI, Alexa-488, Alexa-546 and Alexa-633, respectively. Images were taken through a 10× or a 20× objective. Quantifications were performed with ImageJ 1.36b software (NIH, USA). Briefly, pAkt levels were assessed by measuring the integrated fluorescence densities after background subtraction within nine reproductive contours of dentate gyrus throughout the rostro-caudal and medio-lateral axes of the brain. All the values were normalized with that of the control condition of the same animal and final data represented as the mean percentage of the control ± SEM. Besides, the number of pGSK3β-, Hu- and/or Ki67-positive cells was counted manually in the whole subgranular zone of each dentate gyrus. The data represent the average of the sum of 3 sections distributed homogeneously on the rostro-caudal axis ± SEM.

Measure of depression-like behavior

Anhedonia. Anhedonia was evaluated by performing a saccharin preference test modified from[39]. In order to prevent a possible bias induced by the consumption of a high calorie and low carbohydrate HF diet on the appeal to a high sugar beverage, and to limit the impact of such a beverage on glycaemia, we used saccharin, a palatable sweetener for rats [40], instead of sucrose. Prior to the preference test, rats were submitted to two successive habituations of 2 days each. The first one consisted in training the rats to drink water from two bottles, and the second one aimed to prevent novelty-dependent preference to sweetener by exposing the rats to two bottles filled with 0.025% saccharin. Then the rats were given a choice between water and increasing concentrations (0.025% and 0.05%) saccharin for 2 days per concentration. To avoid placement-dependent preferences, the position of the two bottles was randomly changed

every day. The relative daily saccharin intake was calculated as absolute intake (g) per rat body weight.

Forced swim test (FST). Our protocol was adapted from[41]. Rats were subjected to two swimming sessions, a pre-test of 15 min followed by a 5-min long test 24 h later. Rats were placed individually in a clear, vertical cylindrical container in Plexiglas (height: 60 cm, diameter: 40 cm) filled to a depth of 45 cm with water at 25 °C. The cylinder was rinsed and filled with clean water before each trial. Rats were dried and warmed up immediately after the swim period, and placed into a temporary dry cage so that pre-swim rats are not in direct contact with post-swim rats. The swimming sessions were recorded from the side using a video camera. The latency and duration of climbing, swimming and immobility, as well as the frequency of head shaking, were measured by using ethological software (Observador®, Behavior Scoring Program, Athens, Greece). The higher body weight of HF rats has not biased the swim performance of the rats since individual body weights did not correlate with either immobility, swimming or climbing.

Circular corridor. The general locomotor activity of the rats was evaluated in the following day. This test was used to rule out the possibility that an increase in the immobility time in the FST was due to a general decrease in ambulatory activity of the HF rats. The apparatus consisted of a circular corridor (Imetronic, Pessac, France) in which the outside radius of the inner wall was 32 cm and the inside radius of the outer wall was 60 cm, providing a 14-cm wide running area. Four infrared beams were placed every 90° at a height of 5 cm above the running platform. The locomotor activity was counted when animals interrupted two successive beams and thus had traveled a quarter of the circular corridor. The number of quarter turns, recorded via a computer equipped with appropriate software, was used as an

index of the locomotor activity. The activity scores were recorded every 10 min for a period of one hour.

Statistics

The data are represented as mean ± standard error of the mean (SEM). Statistical analysis was performed using InVivoStat (invivostat.co.uk) and data was tested for outliers using predicted vs. residuals plot test. Data were statistically analyzed using one-way ANOVA (post hoc Bonferroni), repeated measures parametric analysis or paired Student's *t*-test. Statistical significance was set at $p<0.05$.

Results

Effects of the diets on metabolic parameters

A significant overweight in rats fed a HF diet became established from the second week of regimen (+4.7% in HF compared with C rats; p<0.05; Fig. 1A). During the 16 weeks of HF feeding, the mean body weight progressively increased from 377.9 ± 2.5 to 720.5 ± 21.3 g. By comparison, control animals exhibited a lesser augmentation of their body weight (from 368.8 ± 5.1 g to 639.1 ± 9.3 g) (Fig. 1A). The average body weight was significantly increased in HF rats compared with C animals by 8.9% and by 12.7% after 9 and 16 weeks of regimen, respectively (p<0.001) (Fig. 1A). The establishment of overweight in HF rats was associated with a significant 13.6% increase in mean energy intake after 8 weeks of diet (p<0.001), which persists at 15 weeks (Fig. 1B).

The diet substitution of HF with C food from the tenth to the sixteenth week (HF/C group) was associated with a significant slowdown of the body weight gain curve compared with non-restored HF animals (p<0.05). Nevertheless, HF/C rats remained significantly bigger than C animals after 6 weeks of C food restoration (p<0.001; Fig. 1B). The mean energy intake of HF/C rats was similar to that of C animals (Fig. 1B).

In comparison to C group, rats fed a HF diet for 8 weeks exhibited a significant hyperglycaemia (116.9 ± 1.9 mg/dL in HF vs 109.0 ± 1.8 mg/dL in C; p<0.05) (Fig. 1C). After 16 weeks of diet, HF rats and HF/C were significantly hyperglycaemic in comparison to control animals (Fig. 1C).

HF feeding promotes a reversible depressive-like behavior

In order to determine the functional consequences of a HF diet on the development of depressive symptoms, we used two different behavioral tests: the preference to saccharin and the FST. A significant reduction in the consumption of 0.025% saccharin solution over the

first 24 hrs was observed in rats receiving the HF diet for 8 weeks compared to their controls (p<0.05; Fig. 2A). This difference was no more significant on the two following days (not shown). During the FST, rats fed a HF diet for 9 weeks exhibited a significant decrease in swimming time (p<0.05) and a significant increase in immobility time (p<0.05) compared to their controls (Fig. 2B). The climbing time was similar between HF and C rats. Six weeks of diet substitution led to a total normalization of the swimming and the immobility times in the FST (Fig. 2C). It is noteworthy that the HF diet decreased the immobility latency (not shown), and that this behavioural characteristic was reversed by control diet restoration (238.8 s in C vs 38.7 s in HF and 227.8 s in HF/C; p<0.001). The general locomotor activity of the rats was assessed in a circular corridor. No difference was evidenced between C, HF and HF/C groups (Fig. S1). The number of head shakes during the FST was significantly decreased in HF animals in comparison to C (p<0.05; Fig. 2D).

HF feeding impairs serotonin, insulin and leptin signaling in the subgranular neurons of the dentate gyrus

The immunohistochemical detection of p(Ser9)GSK3β and p(Ser473)Akt after a 5-min-stimulation of hippocampal slices with 5-HT (10 µM), leptin (10 nM) or insulin (100 nM) was used to compare the sensitivity of dentate gyrus cells to these factors in C, HF and HF/C rats.

In C rats, the incubation of hippocampal slices with 5-HT, leptin or insulin significantly increased the general level of pGSK3β-immunofluorescence in the molecular and granular layers of the dentate gyrus (Fig. 3A-D). Moreover, the number of pGSK3β-positive cell bodies increased, mostly in the subgranular layer (Fig. 3E-F). The augmentation of pGSK3β-positive cells was estimated to 56% ± 7 (p<0.01), 33% ± 2 (p<0.05) and 25% ± 12 (p<0.05) with 5-HT, leptin or insulin, respectively, as compared with basal (not stimulated) conditions (Fig. 3L). The majority of these cells (70%) were neurons (Fig. S2AB),

as shown by co-detecting pGSK3β and HuC/D proteins (arrowheads; Fig. 3E-G). This indicates that 5-HT, leptin and insulin stimulations increased the absolute number of pGSK3β-positive neurons in the subgranular zone of the dentate gyrus (Fig. S2B). The number of non-neuronal (Hu-negative) pGSK3β-positive cells remained unchanged after the treatments (Fig. S2A). Serotonin, leptin and insulin were no more capable of inducing phosphorylation of GSK3β in the dentate gyrus of HF animals (Fig. 3H-L). On the contrary, the response to these factors of dentate gyrus coming from HF/C rats was not significantly different to that of C rats. Indeed, the average number of pGSK3β cells was significantly increased by $40\% \pm 18$ ($p < 0.01$), $46\% \pm 28$ ($p < 0.05$) and $41\% \pm 20$ ($p < 0.05$) after incubation with 5-HT, leptin or insulin respectively, as compared with basal conditions (Fig. 3L).

The perfusion of hippocampal slices obtained from C rats with 5-HT, leptin or insulin almost trebled pAkt levels in the dentate gyrus (Fig. 4A-D, L). This effect was mainly observed in the subgranular zone of the dentate gyrus. It is noteworthy that some scattered pAkt-positive cells were also located in the hilus. PhosphoAkt was mainly found in neurons, as indicated by co-detecting HuC/D protein (arrowheads; Fig. 4E-G). Hippocampal slices obtained from HF rats exhibited no phosphorylation of Akt in the dentate gyrus after stimulation with 5-HT, leptin or insulin (Fig. 4H-L). The restoration of a C diet for 6 weeks tended to normalize the sensitivity of dentate gyrus cells to 5-HT, leptin and insulin, since these factors induced an augmentation of pAkt levels estimated to $72\% \pm 16$ ($p < 0.01$), $127\% \pm 40$ ($p < 0.001$) and $160\% \pm 32$ ($p < 0.001$), respectively, compared to non-stimulated condition (Fig. 4L). These values were not significantly different to those of C rats.

Depressive-like behavior correlates negatively with serotonin-induced GSK3β phosphorylation in the subgranular neurons of the dentate gyrus.

Taking all the groups into account, the 5-HT-induced phosphorylation of GSK3β in the subgranular cells was correlated positively with the swimming time and negatively with the immobility time (Fig. 5A,D; $p<0.05$). Likewise, the response of subgranular cells to leptin positively correlated with the swimming time (Fig. 5B; $p<0.05$). However, no correlation was found between any behavioural parameters and the insulin-induced phosphorylation of GSK3β (Fig. 5C,F). Similarly, no correlation was evidenced between phosphorylation levels of Akt and FST parameters (Fig. 5G-L). Finally, no correlation was found between phosphorylation of GSK3β/Akt in the subgranular cells with the climbing times (not shown).

Discussion

The current study demonstrates that HF diet-induced metabolic syndrome promotes a reversible depressive-like behavior in rats, which occurs, at least in part, due to an impairment of the Akt/GSK3β pathway activation in the hippocampus. Indeed, we show that, in physiological conditions, leptin and insulin increase the phosphorylation of Akt and GSK3β in the subgranular neurons of the dentate gyrus, and that the activation of this pathway is prevented by a 16-week-long HF diet. Moreover, this HF-induced leptin- and insulin-resistance was associated with a blockade of the serotonin-induced activation of the Akt/GSK3β pathway in the same area, suggesting a possible cross-talk between leptin, insulin and serotonin signaling in the subgranular neurons. These data enrich the understanding of the neurobiological links between metabolic pathologies and depression and propose an alternative mechanism to the ones described previously[24, 25].

GSK3β, a serine/threonine protein kinase and downstream substrate of the PI3K/Akt pathway [21], plays an important role in mood regulation [42], notably in the physiopathology of depression. For instance, heterozygous GSK3β^{+/-} mice are less prone to depression since they exhibit decreased immobility time in the FST [43, 44], and selective GSK3 inhibitors exert antidepressant action in the FST in mice and rats [45, 46]. In addition, GSK3 is an important target of mood stabilizers and antidepressants [42]. It is inhibited by lithium [47], and mice overexpressing mutant forms of GSK3α and/or β on Ser21 and/or Ser9 residues exhibit increased immobility time in the FST after administration of fluoxetine, revealing that the antidepressant action of fluoxetine occurs via GSK3 inhibition in mice [18, 48].

Our experiments indicate for the first time to our knowledge an alteration of serotonin signaling in HF-induced depression, as evidenced by the accurate analysis of the FST. Indeed, in our study, the HF diet induced an increased immobility time and a decreased swimming time whereas the climbing time was unchanged in comparison to C and HF/C groups,

reflecting a reduced action of the serotonergic system [49, 50]. Furthermore, swimming and immobility times correlated with serotonin-dependent GSK3β phosphorylation levels. These findings suggest that serotonin-induced phosphorylation (namely inhibition) of GSK3β in the dentate gyrus is an essential step for the manifestation of reduced depressive behavior and a process that could represent a molecular link between depression and insulin-/leptin-resistance conditions. Besides, a behavioral aspect linked to serotonergic action that has not been detected in previous studies is the number of head shakes in the forced swim test. We have shown that this behavior, which indicates search for an escape and is associated with the activation of the serotonin receptors 1A, 2 and cAMP activation[51, 52], is also decreased in HF rats when compared with control animals. Finally, we report for the first time to our knowledge a decreased immobility latency in HF-induced depressive-like behavior[53].

Feeding rats a HF diet (40% kcal fat) for 8 weeks caused a depressive-like behavior, as attested by two complementary behavioral experiments, the saccharine preference test and the FST. Indeed, the HF fed animals exhibited a lower relative consumption of saccharine as well as a significant increased immobility time paralleled to a decrease in swimming and immobility latency time in the FST. This indicates that a milder protocol than the ones published earlier (~60% kcal fat for 12[1] to 16[2] weeks) is sufficient to trigger depressive-like behavior. The HF diet primarily induced an overweight and a hyperglycemia, two respective indicators of resistance to leptin and insulin. This resistant state was corroborated in the brain at the cellular level since the HF diet totally prevented the phosphorylation of Akt and GSK3β by these hormones on brain slices.

Previous studies have suggested antidepressant effects of leptin and insulin, as well as a potential involvement of the Akt/GSK3β pathway in this process. For instance, leptin receptor deletion from the hippocampus results in depressive-like behaviors in mice [28]. In addition, i.p. injections of leptin reduce depressive-like symptoms and inhibit hippocampal

GSK3β via phosphorylation on Ser9 in rats [23]. In our experiments, the positive correlation between leptin-induced GSK3β phosphorylation levels and the immobility time in the FST provides the missing link between depression and leptin signaling in the dentate gyrus. It is to note that the involvement of the JAK2/STAT3 pathway in the aforementioned effects of leptin is unlikely, as suggested by Scott and colleagues (Elmquist et al., 2009). Besides, the streptozotocin-treated or db/db diabetic mice exhibit lengthened immobility in the FST, which can be partially reversed by insulin treatment [54]. In addition, it has been shown that rosiglitazone, an anti-diabetic compound improving insulin sensitivity by up-regulation of PI3K/Akt pathway [55], exhibits antidepressant properties in control and diabetic (db/db) mice [56]. However, an impairment of locomotor activity has been observed in these models, limiting the interpretation of the results. Further, we evidenced no significant correlation between insulin-induced Akt/GSK3β phosphorylation levels and the immobility time in the FST. This could reflect a not exclusive but synergistic effect of insulin with other factors, such as leptin and serotonin, yet other effects unrelated to depression are possibly mediated by insulin in this region or elsewhere. Altogether, our data show for the first time that in HF-induced leptin-/insulin-resistance, the impaired activation of the Akt/GSK3β pathway in the dentate gyrus may contribute to the onset of depressive-like behavior.

The phosphorylation of Akt and GSK3β induced by incubation of brain slices with leptin, insulin and serotonin in control and HF/C rats mainly occurred in neurons of the subgranular layer, as evidenced by anti-Hu staining. Moreover, the behavioral parameters correlated with the phosphorylation levels of GSK3β specifically in the subgranular zone, but not in the whole dentate gyrus (including the hilus and the other cellular layers). Thus our study indicates that leptin, insulin and serotonin activate the Akt/GSK3β pathway in the subgranular neurons, and that the impact of the HF diet on depression may involve, at least in part, this area. This is in accordance with a previous study showing that injection of lentiviral

GSK3β shRNA in this region that decrease the level of this protein in the subgranular cells decreased immobility times in FST and tail suspension test[57]. Whereas it was known that subgranular neurons express leptin [31, 32], insulin [30] and serotonin receptors [58], we show here for the first time a possible cross-talk could exist between these factors in this area, which would underlie the link between metabolic syndrome and depression. Several brain regions have been proposed to contribute to mood disorders. However, the subgranular layer of the dentate gyrus has been thought to play a major role in the physiopathology of depression. Indeed, this region is submitted to a continuous neurogenesis through lifespan, which is required for the behavioral effects of antidepressants[59]. Such a neurogenesis is modulated by a variety of factors, including serotonin [60], insulin [61] and leptin [23] and involves GSK3β [62, 63].

Taken together, our data indicate that a HF-induced leptin- and/or insulin-resistance may alter mood directly or indirectly through the impairment of serotonin signaling at the level of GSK3β in the dentate gyrus. Such a cross-talk between serotonin and insulin signaling has already been observed in the hypothalamus and in neuroblastoma cells via PI3K/Akt [29]. Moreover, the fact that we (and others) evidenced a molecular link between obesity and depression in rodent strongly indicates that the social and psychological aspects are not the only links between these pathologies. A molecular neurobiological substrate underlies this interaction and should be taken into consideration in treating patients suffering from at least one these pathologies. Finally, the deleterious impact of the HF diet on mood and brain signaling could be reversed after six weeks of standard food restoration. This is in agreement with a meta-analysis by Fabricatore *et al*., where it is suggested that weight loss leads to a decrease of depressive symptoms in obese individuals [64]. Nevertheless, the fact that glycaemia and body weight remained elevated in HF/C animals indicates that the brain

responds faster than peripheral organs to food restoration. This could be of great importance for clinical aspects of psychological disorders related to metabolic disease.

In conclusion, our study shows that in HF-induced metabolic syndrome, leptin and insulin resistance may impact on the serotonin-dependent phosphorylation of GSK3β in the subgranular neurons of the dentate gyrus, which contributes to the onset of depression. Such an alteration is reversible at the molecular and behavioral levels with an appropriate diet. Taken together, our data suggest the existence of a signaling cross-talk between serotonin, leptin and insulin converging on GSK3β, which may underlie the link between metabolic syndrome (including obesity and type 2 diabetes) and depression. The bidirectional association between these pathologies deserves further investigation. More generally, our study suggests that metabolic syndrome and depression should be incorporated into clinical decisions as symptoms of a common pathology.

References

1.   Chen L, Magliano DJ, Zimmet PZ. The worldwide epidemiology of type 2 diabetes mellitus--present and future perspectives. *Nat Rev Endocrinol* 2012; **8**(4)**:** 228-236.

2.   Malik VS, Willett WC, Hu FB. Global obesity: trends, risk factors and policy implications. *Nat Rev Endocrinol* 2013; **9**(1)**:** 13-27.

3.   Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* 2006; **444**(7121)**:** 840-846.

4.   Konner AC, Bruning JC. Selective insulin and leptin resistance in metabolic disorders. *Cell Metab* 2012; **16**(2)**:** 144-152.

5.   Faith MS, Butryn M, Wadden TA, Fabricatore A, Nguyen AM, Heymsfield SB. Evidence for prospective associations among depression and obesity in population-based studies. *Obes Rev* 2011; **12**(5)**:** e438-453.

6.   Nouwen A, Winkley K, Twisk J, Lloyd CE, Peyrot M, Ismail K *et al.* Type 2 diabetes mellitus as a risk factor for the onset of depression: a systematic review and meta-analysis. *Diabetologia* 2010; **53**(12)**:** 2480-2486.

7.   Wittchen HU, Jacobi F, Rehm J, Gustavsson A, Svensson M, Jonsson B *et al.* The size and burden of mental disorders and other disorders of the brain in Europe 2010. *Eur Neuropsychopharmacol* 2011; **21**(9)**:** 655-679.

8.   Kivimaki M, Batty GD, Jokela M, Ebmeier KP, Vahtera J, Virtanen M *et al.* Antidepressant medication use and risk of hyperglycemia and diabetes mellitus: a noncausal association? *Biol Psychiatry* 2011; **70**(10)**:** 978-984.

9.   Kivimaki M, Hamer M, Batty GD, Geddes JR, Tabak AG, Pentti J *et al.* Antidepressant medication use, weight gain, and risk of type 2 diabetes: a population-based study. *Diabetes Care* 2010; **33**(12)**:** 2611-2616.

10.   Shomaker LB, Tanofsky-Kraff M, Stern EA, Miller R, Zocca JM, Field SE *et al.* Longitudinal study of depressive symptoms and progression of insulin resistance in youth at risk for adult obesity. *Diabetes Care* 2011; **34**(11)**:** 2458-2463.

11.   Pan A, Keum N, Okereke OI, Sun Q, Kivimaki M, Rubin RR *et al.* Bidirectional association between depression and metabolic syndrome: a systematic review and meta-analysis of epidemiological studies. *Diabetes Care* 2012; **35**(5)**:** 1171-1180.

12.   Belgardt BF, Bruning JC. CNS leptin and insulin action in the control of energy homeostasis. *Ann N Y Acad Sci* 2010; **1212:** 97-113.

13.   Benomar Y, Roy AF, Aubourg A, Djiane J, Taouis M. Cross down-regulation of leptin and insulin receptor expression and signalling in a human neuronal cell line. *Biochem J* 2005; **388**(Pt 3)**:** 929-939.

14.   Benomar Y, Wetzler S, Larue-Achagiotis C, Djiane J, Tome D, Taouis M. In vivo leptin infusion impairs insulin and leptin signalling in liver and hypothalamus. *Mol Cell Endocrinol* 2005; **242**(1-2)**:** 59-66.

15.   Kim B, Feldman EL. Insulin resistance in the nervous system. *Trends Endocrinol Metab* 2012; **23**(3)**:** 133-141.

16.   Hay N. Akt isoforms and glucose homeostasis - the leptin connection. *Trends Endocrinol Metab* 2011; **22**(2)**:** 66-73.

17.   Belmaker RH, Agam G. Major depressive disorder. *N Engl J Med* 2008; **358**(1)**:** 55-68.

18.   Polter AM, Yang S, Jope RS, Li X. Functional significance of glycogen synthase kinase-3 regulation by serotonin. *Cell Signal* 2012; **24**(1)**:** 265-271.

19.   Li X, Zhu W, Roh MS, Friedman AB, Rosborough K, Jope RS. In vivo regulation of glycogen synthase kinase-3beta (GSK3beta) by serotonergic activity in mouse brain. *Neuropsychopharmacology* 2004; **29**(8)**:** 1426-1431.

20.   Polter AM, Li X. Glycogen Synthase Kinase-3 is an Intermediate Modulator of Serotonin Neurotransmission. *Front Mol Neurosci* 2011; **4:** 31.

21.   Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell* 2007; **129**(7)**:** 1261-1274.

22.   Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 1995; **378**(6559)**:** 785-789.

23.   Garza JC, Guo M, Zhang W, Lu XY. Leptin restores adult hippocampal neurogenesis in a chronic unpredictable stress model of depression and reverses glucocorticoid-induced inhibition of GSK-3beta/beta-catenin signaling. *Mol Psychiatry* 2012; **17**(8)**:** 790-808.

24.   Yamada N, Katsuura G, Ochi Y, Ebihara K, Kusakabe T, Hosoda K *et al.* Impaired CNS leptin action is implicated in depression associated with obesity. *Endocrinology* 2011; **152**(7)**:** 2634-2643.

25.   Sharma S, Fulton S. Diet-induced obesity promotes depressive-like behaviour that is associated with neural adaptations in brain reward circuitry. *Int J Obes (Lond)* 2012.

26.   Abildgaard A, Solskov L, Volke V, Harvey BH, Lund S, Wegener G. A high-fat diet exacerbates depressive-like behavior in the Flinders Sensitive Line (FSL) rat, a genetic model of depression. *Psychoneuroendocrinology* 2011; **36**(5)**:** 623-633.

27.   Grillo CA, Piroli GG, Kaigler KF, Wilson SP, Wilson MA, Reagan LP. Downregulation of hypothalamic insulin receptor expression elicits depressive-like behaviors in rats. *Behav Brain Res* 2011; **222**(1)**:** 230-235.

28.   Guo M, Huang TY, Garza JC, Chua SC, Lu XY. Selective deletion of leptin receptors in adult hippocampus induces depression-related behaviours. *Int J Neuropsychopharmacol* 2012**:** 1-11.

29.   Papazoglou I, Berthou F, Vicaire N, Rouch C, Markaki EM, Bailbe D *et al.* Hypothalamic serotonin-insulin signaling cross-talk and alterations in a type 2 diabetic model. *Mol Cell Endocrinol* 2012; **350**(1)**:** 136-144.

30.   Farrar CE, Huang CS, Clarke SG, Houser CR. Increased cell proliferation and granule cell number in the dentate gyrus of protein repair-deficient mice. *J Comp Neurol* 2005; **493**(4)**:** 524-537.

31.   Scott MM, Lachey JL, Sternson SM, Lee CE, Elias CF, Friedman JM *et al.* Leptin targets in the mouse brain. *J Comp Neurol* 2009; **514**(5)**:** 518-532.

32.   Patterson CM, Leshan RL, Jones JC, Myers MG, Jr. Molecular mapping of mouse brain regions innervated by leptin receptor-expressing cells. *Brain Res* 2011; **1378:** 18-28.

33.   Folli F, Bonfanti L, Renard E, Kahn CR, Merighi A. Insulin receptor substrate-1 (IRS-1) distribution in the rat central nervous system. *J Neurosci* 1994; **14**(11 Pt 1)**:** 6412-6422.

34.   Djavadian RL. Serotonin and neurogenesis in the hippocampal dentate gyrus of adult mammals. *Acta Neurobiol Exp (Wars)* 2004; **64**(2)**:** 189-200.

35.   Vertes RP, Fortin WJ, Crane AM. Projections of the median raphe nucleus in the rat. *J Comp Neurol* 1999; **407**(4)**:** 555-582.

36.   Vacher CM, Gassmann M, Desrayaud S, Challet E, Bradaia A, Hoyer D *et al.* Hyperdopaminergia and altered locomotor activity in GABAB1-deficient mice. *J Neurochem* 2006; **97**(4)**:** 979-991.

37.   Vacher CM, Hardin-Pouzet H, Steinbusch HW, Calas A, De Vente J. The effects of nitric oxide on magnocellular neurons could involve multiple indirect cyclic GMP-dependent pathways. *Eur J Neurosci* 2003; **17**(3)**:** 455-466.

38.   Couvreur O, Aubourg A, Crepin D, Degrouard J, Gertler A, Taouis M *et al.* The anorexigenic cytokine ciliary neurotrophic factor stimulates POMC gene expression via receptors localized in the nucleus of arcuate neurons. *Am J Physiol Endocrinol Metab* 2012; **302**(4)**:** E458-467.

39.   Cope EC, Morris DR, Scrimgeour AG, VanLandingham JW, Levenson CW. Zinc supplementation provides behavioral resiliency in a rat model of traumatic brain injury. *Physiol Behav* 2011; **104**(5)**:** 942-947.

40.   Sclafani A, Abrams M. Rats show only a weak preference for the artificial sweetener aspartame. *Physiol Behav* 1986; **37**(2)**:** 253-256.

41.   Slattery DA, Cryan JF. Using the rat forced swim test to assess antidepressant-like activity in rodents. *Nat Protoc* 2012; **7**(6)**:** 1009-1014.

42.   Li X, Jope RS. Is glycogen synthase kinase-3 a central modulator in mood regulation? *Neuropsychopharmacology* 2010; **35**(11)**:** 2143-2154.

43.   Ackermann TF, Kempe DS, Lang F, Lang UE. Hyperactivity and enhanced curiosity of mice expressing PKB/SGK-resistant glycogen synthase kinase-3 (GSK-3). *Cell Physiol Biochem* 2010; **25**(6)**:** 775-786.

44.   O'Brien WT, Harper AD, Jove F, Woodgett JR, Maretto S, Piccolo S *et al.* Glycogen synthase kinase-3beta haploinsufficiency mimics the behavioral and molecular effects of lithium. *J Neurosci* 2004; **24**(30)**:** 6791-6798.

45.   Kaidanovich-Beilin O, Milman A, Weizman A, Pick CG, Eldar-Finkelman H. Rapid antidepressive-like activity of specific glycogen synthase kinase-3 inhibitor and its effect on beta-catenin in mouse hippocampus. *Biol Psychiatry* 2004; **55**(8)**:** 781-784.

46.   Gould TD, Einat H, Bhat R, Manji HK. AR-A014418, a selective GSK-3 inhibitor, produces antidepressant-like effects in the forced swim test. *Int J Neuropsychopharmacol* 2004; **7**(4)**:** 387-390.

47.   Klein PS, Melton DA. A molecular mechanism for the effect of lithium on development. *Proc Natl Acad Sci U S A* 1996; **93**(16)**:** 8455-8459.

48.   Polter A, Beurel E, Yang S, Garner R, Song L, Miller CA *et al.* Deficiency in the inhibitory serine-phosphorylation of glycogen synthase kinase-3 increases sensitivity to mood disturbances. *Neuropsychopharmacology* 2010; **35**(8)**:** 1761-1774.

49.   Detke MJ, Lucki I. Detection of serotonergic and noradrenergic antidepressants in the rat forced swimming test: the effects of water depth. *Behav Brain Res* 1996; **73**(1-2)**:** 43-46.

50.   Page ME, Detke MJ, Dalvi A, Kirby LG, Lucki I. Serotonergic mediation of the effects of fluoxetine, but not desipramine, in the rat forced swimming test. *Psychopharmacology (Berl)* 1999; **147**(2)**:** 162-167.

51.   Drossopoulou G, Antoniou K, Kitraki E, Papathanasiou G, Papalexi E, Dalla C *et al.* Sex differences in behavioral, neurochemical and neuroendocrine effects induced by the forced swim test in rats. *Neuroscience* 2004; **126**(4)**:** 849-857.

52.   Wachtel H. Characteristic behavioural alterations in rats induced by rolipram and other selective adenosine cyclic 3', 5'-monophosphate phosphodiesterase inhibitors. *Psychopharmacology (Berl)* 1982; **77**(4)**:** 309-316.

53.   Pliakas AM, Carlson RR, Neve RL, Konradi C, Nestler EJ, Carlezon WA, Jr. Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element-binding protein expression in nucleus accumbens. *J Neurosci* 2001; **21**(18)**:** 7397-7403.

54.   Hilakivi-Clarke LA, Wozniak KM, Durcan MJ, Linnoila M. Behavior of streptozotocin-diabetic mice in tests of exploration, locomotion, anxiety, depression and aggression. *Physiol Behav* 1990; **48**(3)**:** 429-433.

55.   Standaert ML, Kanoh Y, Sajan MP, Bandyopadhyay G, Farese RV. Cbl, IRS-1, and IRS-2 mediate effects of rosiglitazone on PI3K, PKC-lambda, and glucose transport in 3T3/L1 adipocytes. *Endocrinology* 2002; **143**(5)**:** 1705-1716.

56.   Sharma AN, Elased KM, Lucot JB. Rosiglitazone treatment reversed depression- but not psychosis-like behavior of db/db diabetic mice. *J Psychopharmacol* 2012; **26**(5)**:** 724-732.

57.   Omata N, Chiu CT, Moya PR, Leng Y, Wang Z, Hunsberger JG *et al.* Lentivirally mediated GSK-3beta silencing in the hippocampal dentate gyrus induces antidepressant-like effects in stressed mice. *Int J Neuropsychopharmacol* 2011; **14**(5)**:** 711-717.

58.   Berumen LC, Rodriguez A, Miledi R, Garcia-Alcocer G. Serotonin receptors in hippocampus. *ScientificWorldJournal* 2012; **2012:** 823493.

59.   Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S *et al.* Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* 2003; **301**(5634)**:** 805-809.

60.   Banasr M, Hery M, Printemps R, Daszuta A. Serotonin-induced increases in adult cell proliferation and neurogenesis are mediated through different and common 5-HT receptor subtypes in the dentate gyrus and the subventricular zone. *Neuropsychopharmacology* 2004; **29**(3)**:** 450-460.

61.   Stranahan AM, Arumugam TV, Cutler RG, Lee K, Egan JM, Mattson MP. Diabetes impairs hippocampal function through glucocorticoid-mediated effects on new and mature neurons. *Nat Neurosci* 2008; **11**(3)**:** 309-317.

62.   Eom TY, Jope RS. Blocked inhibitory serine-phosphorylation of glycogen synthase kinase-3alpha/beta impairs in vivo neural precursor cell proliferation. *Biol Psychiatry* 2009; **66**(5)**:** 494-502.

63.   Kim WY, Wang X, Wu Y, Doble BW, Patel S, Woodgett JR *et al.* GSK-3 is a master regulator of neural progenitor homeostasis. *Nat Neurosci* 2009; **12**(11)**:** 1390-1397.

64.   Fabricatore AN, Wadden TA, Higginbotham AJ, Faulconbridge LF, Nguyen AM, Heymsfield SB *et al.* Intentional weight loss and changes in symptoms of depression: a systematic review and meta-analysis. *Int J Obes (Lond)* 2011; **35**(11)**:** 1363-1376.

Figure legends

Figure 1: Experimental schedule and effect of high-fat diet on body weight, energy intake and glycemia in male Wistar rats. (A) Body weight curves of rats exposed to chow diet (C, closed boxes), high-fat diet (HF, open circles) or high-fat diet reversed to chow diet (HF/C,

closed circles). Data are presented as mean ± SEM. C vs HF: *p<0.05, ***p<0.001, HF vs HF/C: #p<0.05, C vs HF/C: †††p<0.001 (assessed by repeated measures parametric analysis test). The dashed line indicates the starting time of diet restoration. The bars indicate the time of performance of each test. SPT, Saccharin Preference Test; FST, Forced Swim Test. (B) Energy intake curves of rats exposed to chow diet (C, closed boxes), high-fat diet (HF, open circles) or high-fat diet reversed to chow diet (HF/C, closed circles). Mean individual food intake in week 8 and week 15. Data are presented as mean ± SEM. C vs HF: ***p<0.001, HF vs HF/C: **p<0.01, C vs HF/HF: **p<0.01 (assessed by repeated measures parametric analysis test) C: n=10, HF: n=9, HF/HF: n=4, HF/C: n=5 (C) Blood glucose levels of rats exposed to chow diet (C, black), high-fat diet (HF, white) or high-fat diet reversed to chow diet (HF/C, striped) at 8 and 16 weeks. C: n=10, HF: n=9, HF/HF: n=4, HF/C: n=5. Data are represented as mean ± SEM. *p<0.05 vs C.

Figure 2: Effect of high-fat diet on depressive-like behavior in male Wistar rats. (A) Saccharin preference test in rats exposed for 8 weeks to chow diet (C, black) or high-fat diet (HF, white). (B) Climbing, swimming and immobility times of rats exposed to chow diet (C, black) or high-fat diet (HF, white) for 9 weeks. (C) Climbing, swimming and immobility times of rats exposed to chow diet (C, black), high-fat diet (HF, white) or high-fat diet reversed to chow diet (C, striped) after 16 weeks of experiment. (D) Number of head shakes of rats in the FST exposed to chow diet, high-fat diet or high-fat diet after 8 weeks of experiment. C: n=10, HF: n=9, HF/HF: n=4, HF/C: n=5. All data are presented as mean ± SEM. *p<0.05 vs C. Assessed by one-way ANOVA.

Figure 3: Immunohistochemical detection of p(Ser9)GSK3β (green) and HuC/D (red) in the dentate gyrus. (A-D) Immunofluorescent staining of pGSK3β in rats fed a chow diet after stimulation of brain slices with 5-HT (10 µM), leptin (10 nM) and insulin (100 nM) for 5 min. (E-G) Colocalization of pGSK3β (green) and HuC/D (red) in subgranular cells are shown with arrowheads. (H-K) pGSK3β immunofluorescence in the dentate gyrus of rats fed a HF diet after stimulation of brain slices with 5-HT (10 µM), leptin (10 nM) and insulin (100 nM). For 5 min (L) Quantification. Effect of stimulation with 5-HT, leptin and insulin on the number of pGSK3β-positive cells in the dentate gyrus of rats fed a chow diet (C), high fat (HF) or high-fat diet reversed to chow diet (HF/C). n=3-4 per group. All data are presented as % of control (not stimulated) ± SEM. *p< 0.05; **p< 0.01 vs control (not stimulated). Corresponding DAPI counterstained sections (blue) are shown on the right top corner of each picture. Confocal laser scanning. Focal planes. Scale bars=135 µm (A-D;H-K); 225 µm (E-G).

Figure 4: Immunohistochemical detection of p(Ser473)Akt (green) and HuC/D (red) in the dentate gyrus (A-D) Immunofluorescent staining of pAkt in rats fed a chow diet after stimulation of brain slices with 5-HT (10 µM), leptin (10 nM) and insulin (100 nM) for 5 min. (E-G) Colocalization of pAkt (green) and HuC/D (red) in subgranular cells are shown with arrowheads. (H-K) pAkt immunofluorescence in the dentate gyrus of rats fed a HF diet after stimulation of brain slices with 5-HT (10 µM), leptin (10 nM) and insulin (100 nM) for 5 min. (L) Quantification. Effect of stimulation with 5-HT, leptin and insulin on the integrated density of pAkt immunofluorescence in the dentate gyrus of rats fed a chow diet (C), high fat

(HF) or high-fat diet reversed to chow diet (HF/C). n=4 per group. All data are presented as % of control (not stimulated) ± SEM. *p< 0.05; **p< 0.01 vs control (not stimulated). Corresponding DAPI counterstained sections (blue) are shown on the right top corner of each picture. Confocal laser scanning. Focal planes. Scale bars=135 µm (A-D;H-K); 225 µm (E-G).

Figure 5: Correlation graphs of swimming and immobility times in the FST with GSK3β and Akt phosphorylation in rats after 16 weeks of experiment. Significant correlations of 5-HT-induced GSK3β phosphorylation with (A) swimming time, (B) immobility time, (C) leptin-induced pGSK3β.

Fig. S1. Locomotor activity analysis. Number of quarter turns in the circular corridor over 60 min of rats exposed to chow diet (C, closed boxes), high-fat diet (HF, open circles) or high-fat diet reversed to chow diet (HF/C, closed circles) after 16 weeks of experiment.

Fig. S2. (A) Repartition of HuC/D-positive (Hu+) and negative (Hu-) cells among pGSK3β positive cells in the dentate gyrus of rats (all groups included), after stimulation with 5-HT (10 µM), leptin (10 nM) and insulin (100 nM) for 5 min. (B) Percentage of HuC/D-positive (Hu+) and negative (Hu-) cells among pGSK3β-positive cells in the dentate gyrus of rats (all groups and stimulations included).
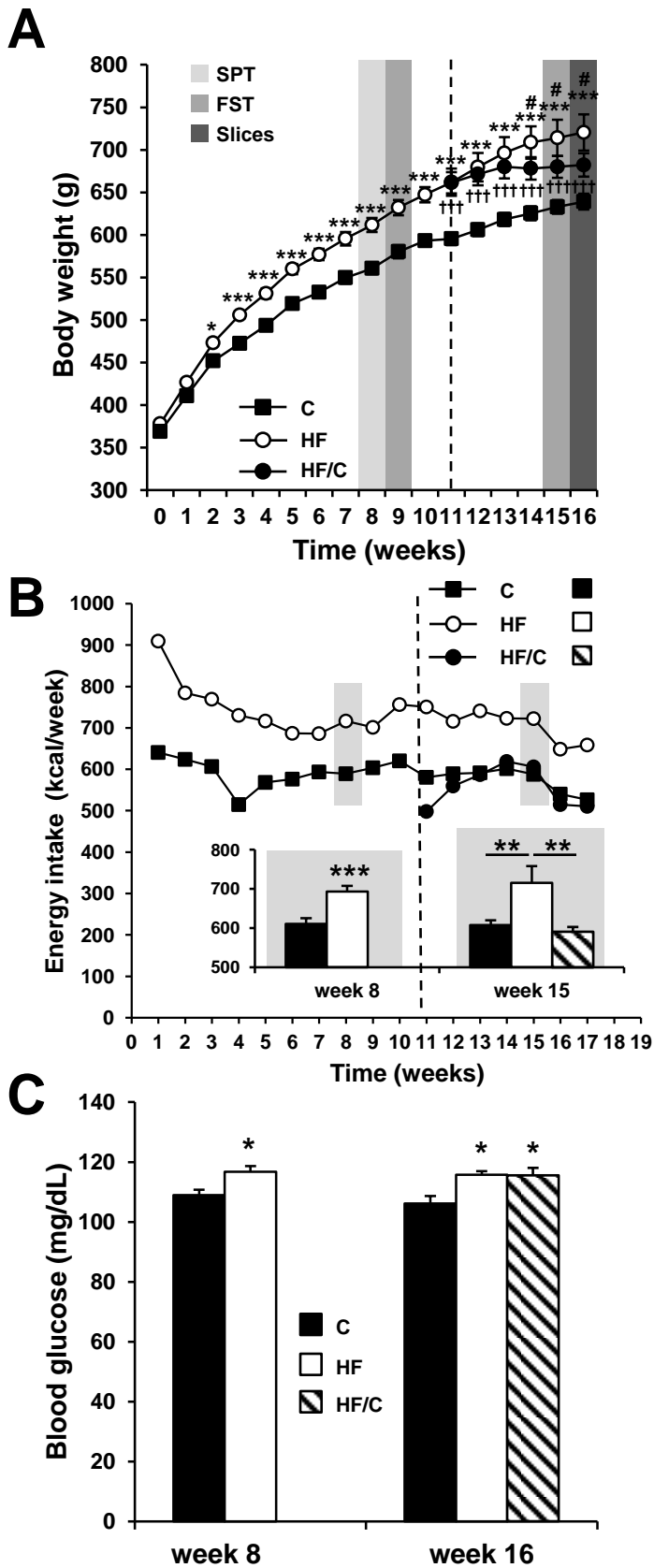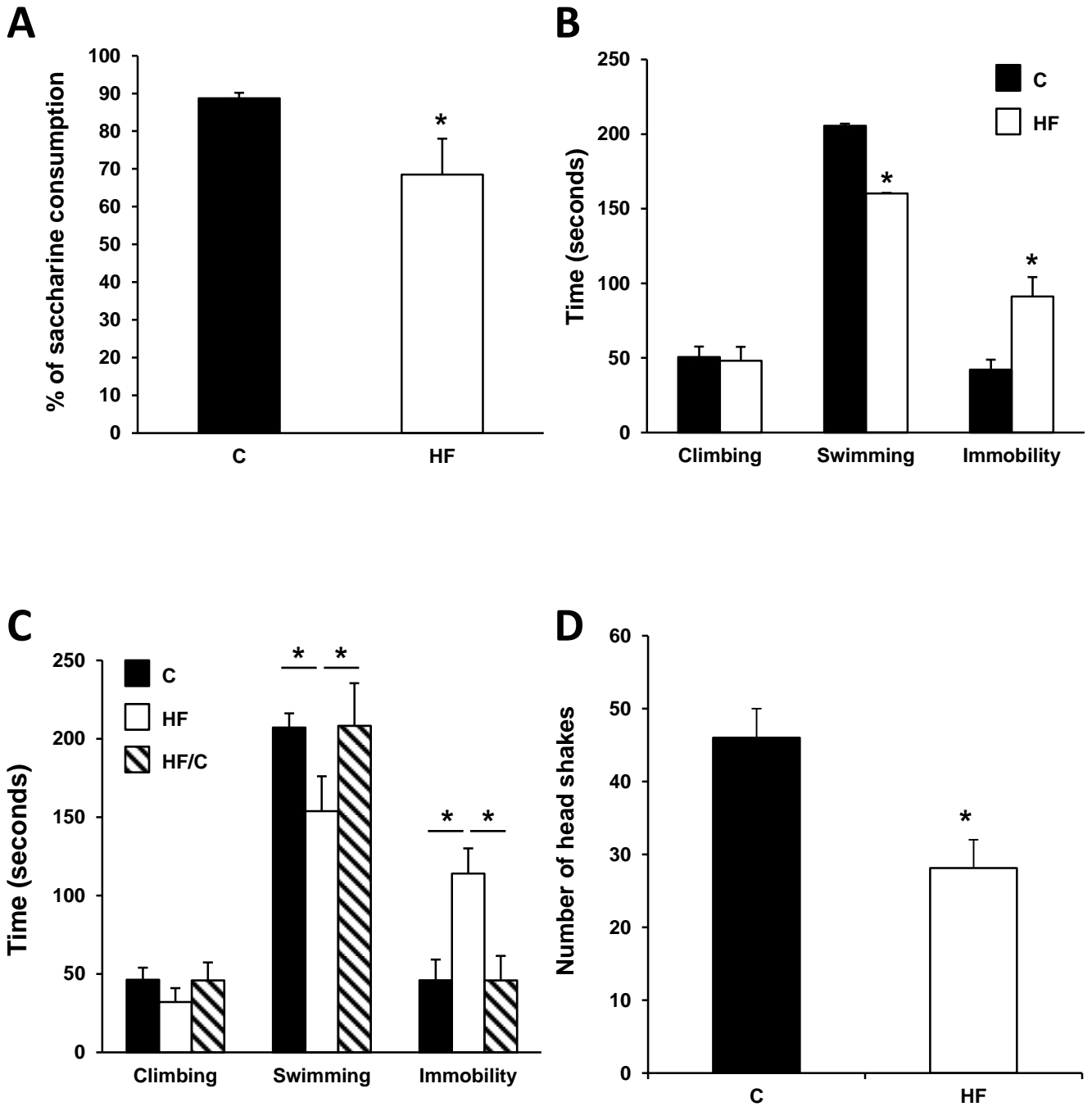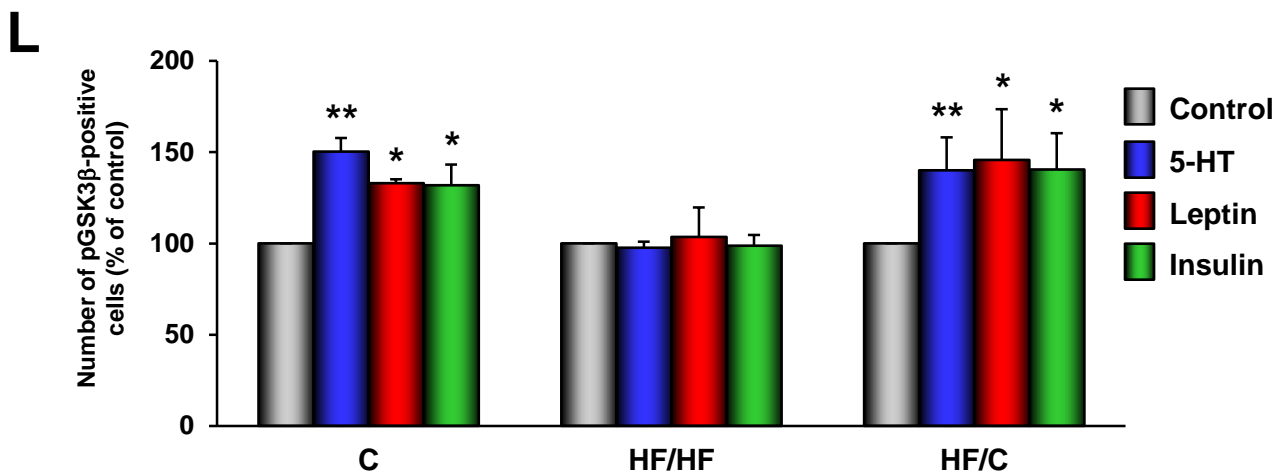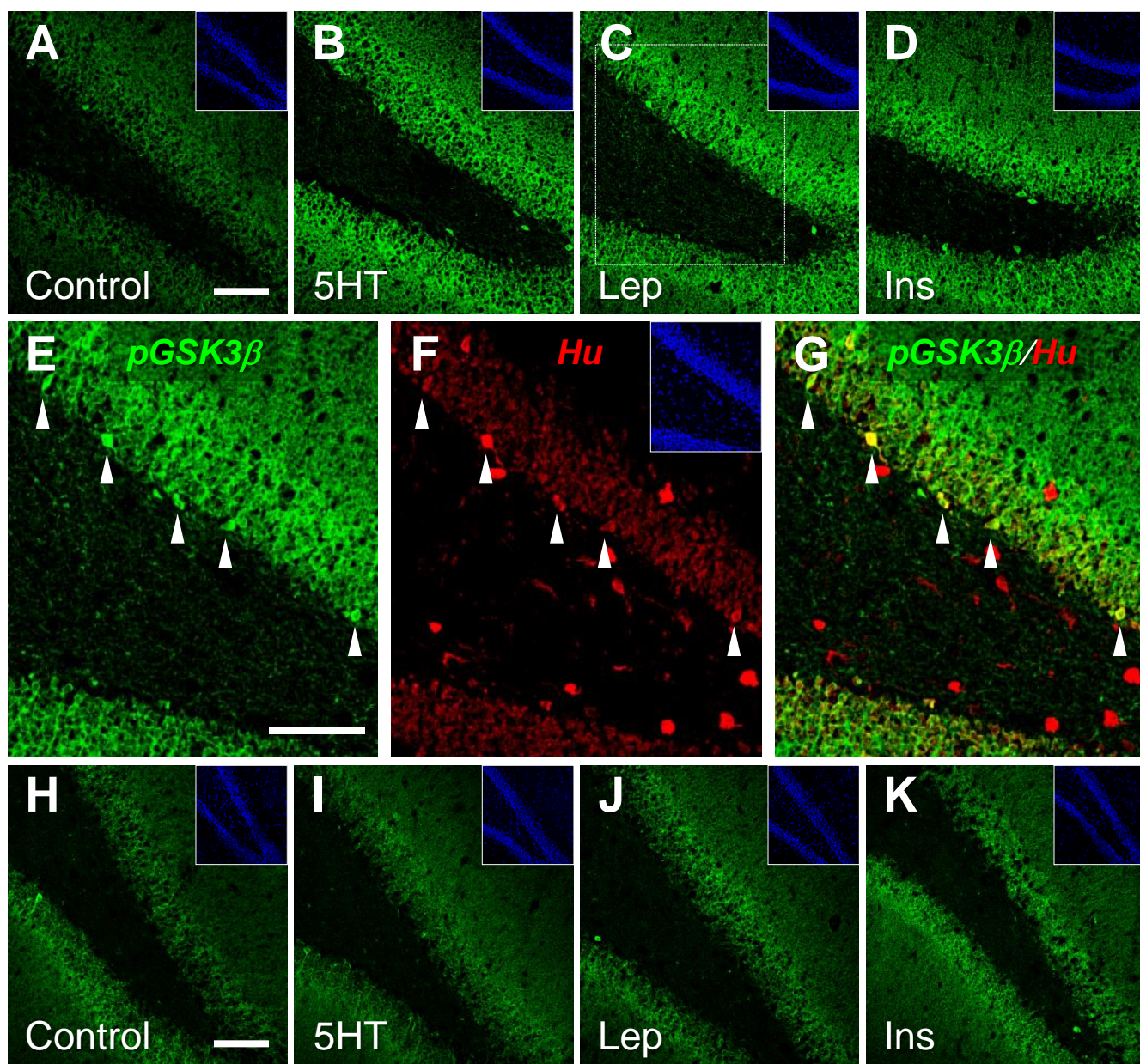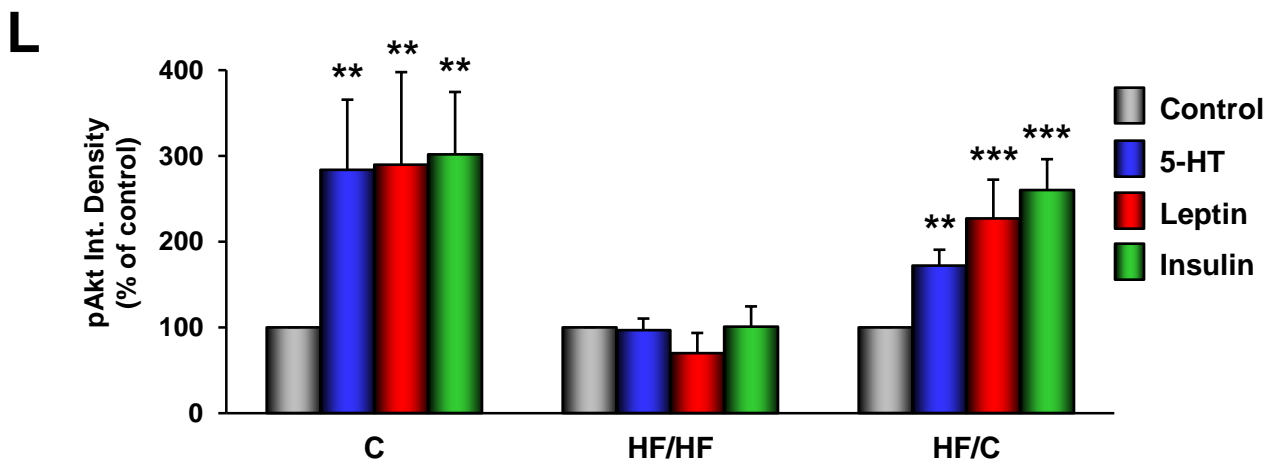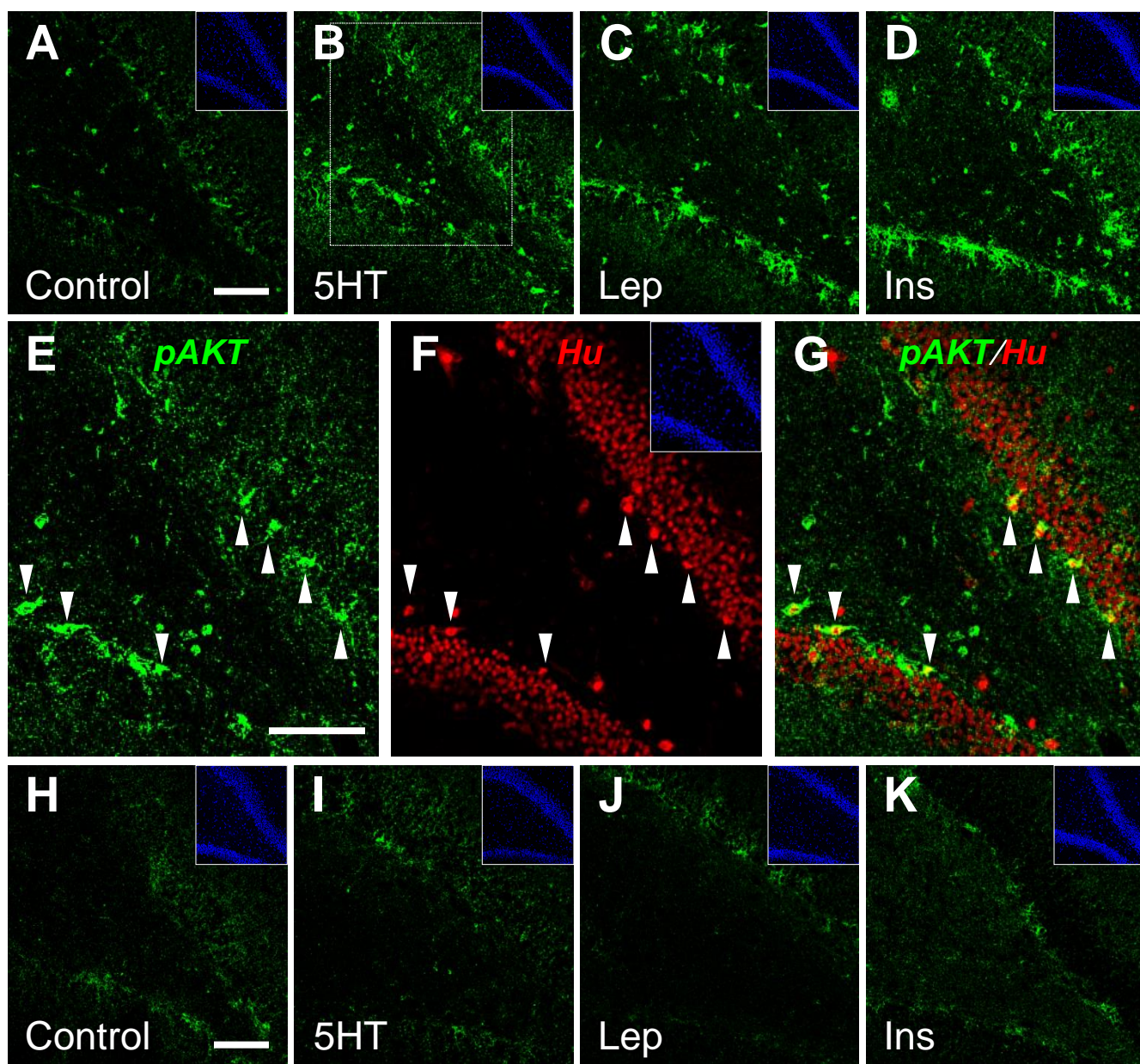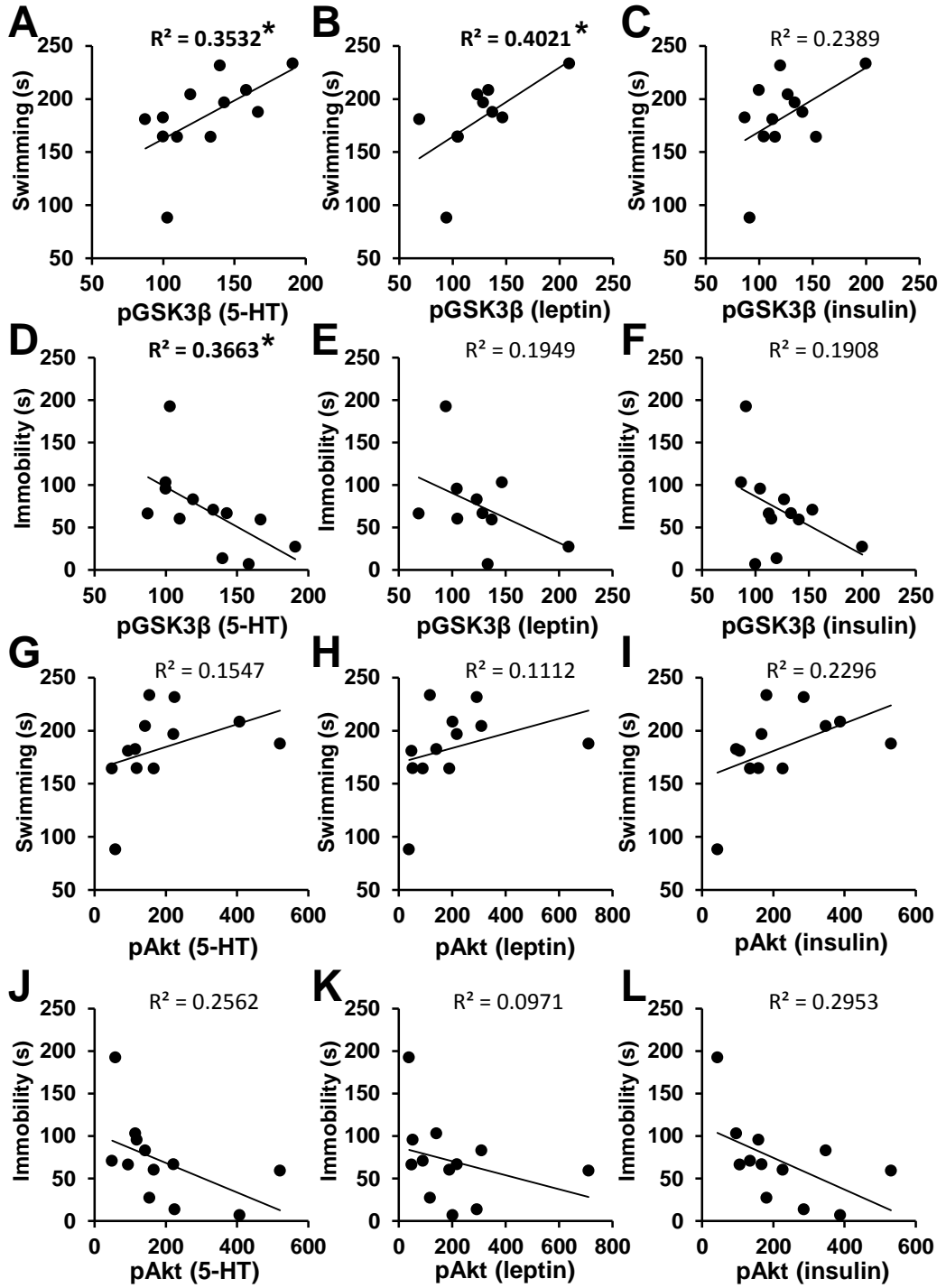
Figure 1

Figure 2

Figure 3

Figure 4

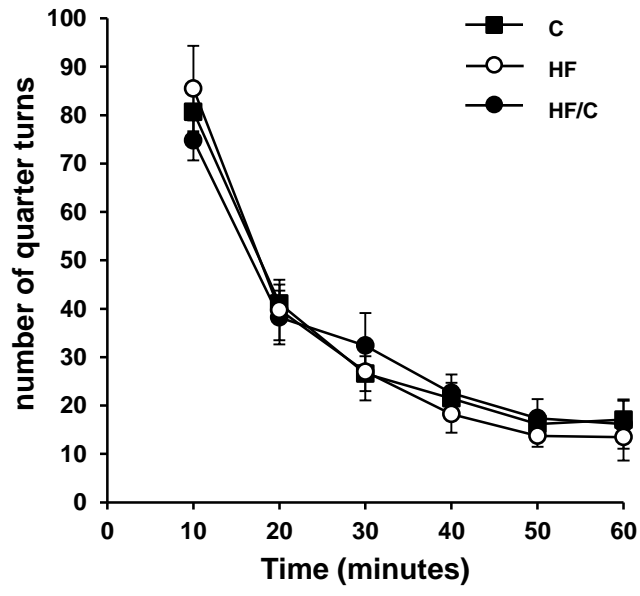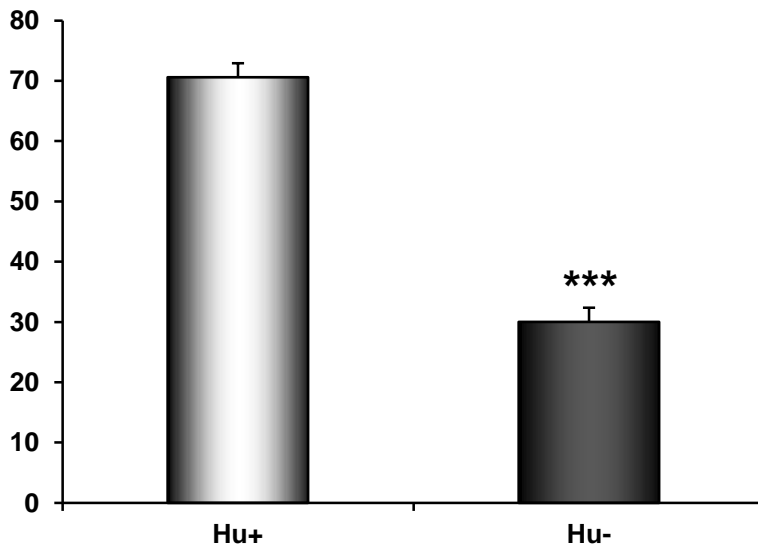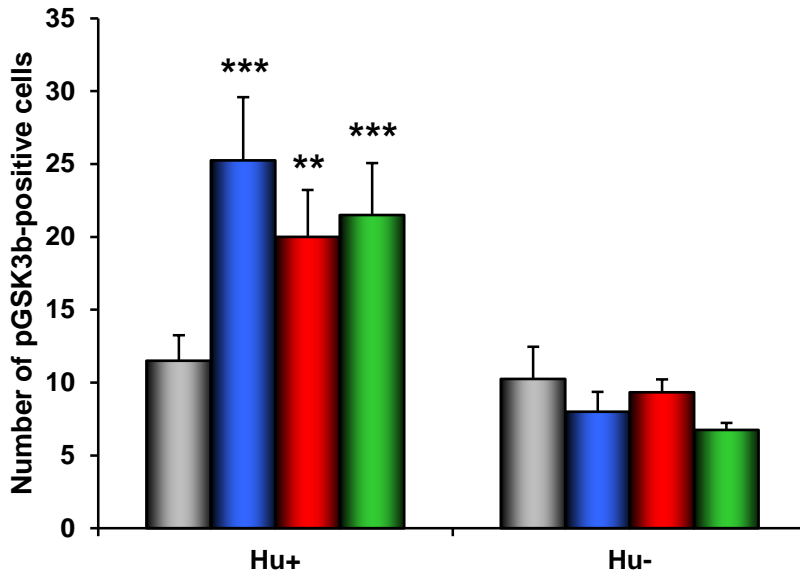# Figure 5

Supplemental data

Figure S1

Figure S2

DISCUSSION

Discussion

Serotonin activates the PI3K/Akt pathway in the hypothalamus

One of our major findings was that dexfenfluramine injection induces an increase in Akt phosphorylation levels in the hypothalamus. Therefore, elevated concentration of serotonin in the hypothalamus results in the stimulation of one or more of its numerous receptors that trigger signaling cascades involving Akt as one of their downstream molecules. As previously reported, several 5-HT receptors are able to initiate the activation of Akt in neuronal cells (Cowen, 2007). However, so far there was no evidence of this serotonergic effect in the hypothalamus. Serotonin release in the rat hypothalamus is well-acknowledged since serotonergic projections from the dorsal raphe reach numerous hypothalamic nuclei such as ARC, PVN, VMH and SCN (Azmitia and Segal, 1978; Willoughby and Blessing, 1987).

Hypothalamic action of serotonin plays an important role in the regulation of energy homeostasis, mainly by modulating feeding behavior (Donovan and Tecott, 2013; Leibowitz et al., 1990). Indeed, a majority of anti-obesity drugs that target the brain stimulate the serotonergic system (e.g. dexfenfluramine, norfenfluramine, benfluorex and sibutramine). They induce an anorectic effect in animals (Rowland, 1986) and humans (Hill and Blundell, 1990). Thus, we hypothesize that the effect of increased serotonin levels on food intake could, at least in part, occur through the activation of PI3K/Akt signaling pathway in appetite regulating neurons. In addition, regulation of peripheral functions such as hepatic insulin sensitivity (Storlien et al., 1989) , hepatic glucose production(Picarel-Blanchot et al., 1994) and energy expenditure (Rothwell and Stock, 1987) by hypothalamic serotonin action, could be possibly initiated via this pathway.

Akt exerts its kinase activity on a plethora of downstream molecules (Manning and Cantley, 2007). One of its substrates involved in hypothalamic energy regulation is the Forkhead box protein O1 (FOXO1), a transcription factor, that down-regulates

POMC and up-regulates AgRP in the ARC (Iskandar et al., 2010; Kim et al., 2006; Sasaki and Kitamura, 2010).In mice, 5-HT has been reported to induce FOXO1 phosphorylation and its translocation from the nucleus to the cytoplasm in the cortex, the hippocampus and the striatum in a PI3K/Akt-dependent manner (Polter et al., 2009). In addition, 5-HT$_{2C}$ receptor activation results in an increase of POMC expression in the ARC (Lam et al., 2008). However, 5-HT does not alter the expression of hypothalamic AgRP (Li et al., 2000). Thus, hypothalamic 5-HT$_{2C}$/PI3K/Akt/FOXO1 pathway could be a possible mediator of the 5-HT action in the regulation of energy homeostasis, and this needs more investigations to decipher the implicated molecular mechanisms in this regulation.

Another Akt direct substrate that plays an important role in the control of energy homeostasis by acting in the hypothalamus is glycogen synthase kinase-3β (GSK3β) (Benzler et al., 2012; Manning and Cantley, 2007). GSK3β inhibitor delivered by i.c.v. ameliorates hypothalamic Akt signaling and glucose tolerance in obese ob/ob mice while overexpression of GSK3β in the mediobasal hypothalamus of wild type mice results in increased food intake and body weight and aggravates the toxic effects of high-fat diet (Benzler et al., 2012). Similar outcomes have been described from mice treated with a 5-HT$_{2C}$ receptor agonist (Zhou et al., 2007) and in 5-HT$_{2C}$ KO/mutant mice respectively (Nonogaki et al., 1998; Tecott et al., 1995). 5-HT is a major regulator of GSK3β in the brain via the activation of different receptors with equivalent or opposing actions, demonstrated with administration of dexfenfluramine, agonists and antagonists of 5-HT receptors (1A,2A/2C) (Li et al., 2004). These data suggest that Akt phosphorylation by 5-HT could play an important role in the overall glucose metabolism and the regulation of feeding behavior.

However, to clearly demonstrate the importance of neural Akt signaling pathway in serotonin-dependent energy homeostasis regulation it is necessary to develop Akt genetic models with specific KO in the whole brain or distinguished neurons. So far, these models are not yet available. The only Akt genetic models available concern single or double global Akt1 or Akt2 knockouts (Hay, 2011). Fortunately, valuable data were

obtained from studies with deletions, mutations, overexpression or repression of other insulin signaling pathway components e.g. receptors (Balthasar et al., 2004; Konner et al., 2007; Xu et al., 2010), PI3K isoforms (Al-Qassab et al., 2009), PDK (Iskandar et al., 2010), TSC1 (Yang et al., 2012), FOXO1 (Fukuda et al., 2008).

The hypothalamic suprachiasmatic nucleus (SCN) receives robust serotonergic innervation, from both dorsal and median raphe, and could account for the response that we observed after the administration of dexfenfluramine (Steinbusch, 1981). In mammals, this region is considered as the "master clock" due to its crucial role in the orchestration of circadian rhythms and hormone secretion (Kriegsfeld and Silver, 2006). 5-HT is an important regulator of the circadian cycles in the SCN mainly through the activation of 5-HT$_{1A}$ and 5-HT$_7$ receptors (Moriya et al., 1998). 5-HT$_{1A}$ agonist 8-OH-DPAT also induces an increase of melatonin receptors which is reversed by the antagonist NAN-190 (Recio et al., 1996). Melatonin receptor stimulation in the rat SCN activates the insulin signaling cascade including IRS1, PI3K, Akt and ERK1/2 (Anhe et al., 2004). Recently, it was demonstrated that when high fat diet feeding is restricted during the dark phase in mice, it prevents from diet-induced obesity and insulin resistance, suggesting the significance of the circadian rhythms in the regulation of metabolism (Hatori et al., 2012). Direct activation of Akt by 5-HT has not been reported in the SCN. However, a similar mechanism to melatonin GPCR could, at least partially, explain the increase in Akt phosphorylation that we observed and the impairment of this effect in the hypothalamus of diabetic rats. The activation of Akt by 5-HT and insulin using brain slices would elucidate the potential role of this pathway in the metabolism regulation by the SCN.

Conclusively, a further dissection of the Akt activation by 5-HT in the hypothalamus is necessary for unraveling the roles of this function in the brain nuclei mediating serotonergic signals of energy homeostasis and feeding regulation.

Serotonin activates PI3K/Akt pathway in SH-SY5Y cells

In the human neuronal cell line SH-SY5Y, we demonstrated that the serotonin induces PI3K-dependent Akt phosphorylation (Papazoglou et al., 2012). Serotonin-induced activation of Akt has been demonstrated to occur in both neuronal and non-neuronal cells (Cowen, 2007).

Cross talk between serotonin and insulin in the SH-SY5Y human neuroblastoma cells

In one of the principal studies we demonstrated the possibility of a cross-talk between insulin and serotonin signaling pathways. More precisely in differentiated SH-SY5Y neuroblastoma cells that underwent insulin-pretreatment in order to mimic insulin resistance conditions, serotonin-induced Akt phosphorylation was impaired (Papazoglou et al., 2012). In this case, insulin signaling pathways are down-regulated leading to the loss of both insulin and leptin responsiveness (Benomar et al., 2005a). On the other hand, chronic treatment with 5-HT (Li et al., 2012) or SSRIs (Levkovitz et al., 2007) induces insulin resistance in 3T3-L1 cells and rat hepatoma Fao cells respectively.

Interestingly, we noticed also that when administered together, 5-HT and insulin, introduce an additive effect in terms of Akt phosphorylation. This result could be explained either by direct interactions of the 5-HT receptors with the insulin receptor as mentioned earlier or by 5-HT-induced inhibition of protein kinases that down-regulate the insulin signaling pathway. Such protein is GSK3β which is constantly active and phosphorylates IRS1 (Liberman and Eldar-Finkelman, 2005) and IRS2 (Sharfi and Eldar-Finkelman, 2008) on Ser^{332} and $Ser^{484, 488}$ respectively, resulting in decreased IRS/PI3K association and insulin signaling activation (Summers et al., 1999). 5-HT-induced phosphorylation of GSK3β (Polter and Li, 2011) can thus enhance the initiation of insulin signals leading to Akt phosphorylation. Equally, insulin-induced GSK3β inhibition (van Weeren et al., 1998) can enhance the activation and cell surface deposition of 5-HT$_{1B}$ (Chen et al., 2009), one of the receptors found to activate Akt (Leone et al., 2000). In

addition, the phosphatase PP2A that dephosphorylates Akt on Thr[308] leading to its inactivation could also be involved in insulin/5HT cross-talk. Indeed, PP2A is inhibited by insulin (Srinivasan and Begum, 1994). On the other hand, 5-HT activates the β-arrestin2/Src/Akt via 5-HT$_{2A}$ (Schmid and Bohn, 2010), a pathway that is negatively regulated by PP2A (Beaulieu et al., 2005). Thus, both sides can improve reciprocal sensitivities to activation of this pathway.

Cross-talk between serotonin and insulin in the hypothalamus of rat

In my first article we described the attenuated ability of dexfenfluramine to activate Akt in the hypothalamus of diabetic GK rats. As stated already, one possible interpretation is the transactivation of insulin receptor by 5-HT receptor/s which is decreased in the diabetic rat in comparison to the control.

A direct Akt substrate, FOXO1, is phosphorylated in neurons by administration of both insulin (Fukuda et al., 2008) and dexfenfluramine (Polter et al., 2009) suggesting it as a candidate of common action. FOXO1 is rapidly phosphorylated by Akt and translocated out of the nucleus (Biggs et al., 1999). In the hypothalamus, nuclear FOXO1 plays an essential role in the regulation of energy homeostasis (Kim et al., 2012; Ren et al., 2012; Ropelle et al., 2009). The regulation of food intake requires the activation of the PI3K/Akt/FOXO1 pathway in both POMC (Belgardt et al., 2008; Iskandar et al., 2010) and AgRP (Cao et al., 2011) neurons of the ARC. FOXO1 activation in POMC and AgRP neurons by 5-HT might explain why animal models with specific deletions of IR, LepRb or both receptors in these neurons do not display the predicted phenotype (Balthasar et al., 2004; Hill et al., 2010; Konner et al., 2007). Mice with specific deletion of 5-HT receptors in these neurons are required in order to elucidate if indeed the activation of FOXO1 by 5-HT is an important step for the control of food intake.

In the VMN, infusion of serotonin (Fetissov and Meguid, 2010) or mCPP (Hikiji et al., 2004) regulates food intake while injection of anti-insulin antibodies induce hyperphagia in rats (Strubbe and Mein, 1977). However, mice with selective deletion of

insulin receptor from a group of neurons in this area (SF-1) resulted in decreased deleterious effects of high fat diet (Klockener et al., 2011). This indicates that other neurons are also responsible for the anorexic effect of insulin in this brain region which will probably be targeted by 5-HT too. FOXO1 deletion from the same neurons resulted in decreased body weight, due to increased energy expenditure, and improved glucose tolerance, suggesting that the 5-HT-induced hypophagia in the VMN is not mediated by these neurons or this pathway simultaneously (Kim et al., 2012).

Moreover, mice lacking the serotonin transporter gene (SERT), inferring a chronic increase in serotonin levels both centrally and peripherally, exhibited decreased insulin sensitivity in peripheral tissues and eventually obesity (Chen et al., 2012). This finding suggests that extended activation of the PI3K/Akt pathway by 5-HT in the brain and probably peripheral insulin-sensitive tissues, leads to feedback down-regulation of this pathway in a manner similar to hyperinsulinemia conditions. This existence of a cross-talk between serotonin and insulin is also supported by studies evidencing a long-term synergistic effect of drugs targeting the serotonergic system, such as sibutramine, and the improvement of insulin sensitivity through lifestyle modifications and structured diet (Albu et al., 2010; Ryan, 2004).

Another debatable notion is the co-expression of the receptors in the same neurons in order to be able to have an interaction of signaling pathways. As previously reported in the arcuate nucleus, the effects of insulin and leptin occur in two distinct subpopulations of POMC neurons, demonstrating the lack of crosstalk between these hormones in these cells (Williams et al., 2010). Similarly, POMC neurons expressing $5HT_{2C}$ receptors compose a population that does not express leptin receptors increasing this way the diversity of properties and function of these anorexigenic neuronal groups (Sohn et al., 2011). However, it is yet to be clarified if insulin and $5HT_{2C}$ receptors are located in the same POMC neurons.

5-HT, Insulin and Leptin activate the PI3K/Akt/GSK3β in the dentate gyrus

In the second study included in this thesis, we demonstrated that 5-HT, insulin and leptin stimulation increased the phosphorylation levels of Akt and GSK3β in the dentate gyrus of the hippocampus *ex vivo* on rat brain slices.

Hippocampal GSK3β inhibition by Ser9 phosphorylation has been shown to be directly induced by serotonin (Li et al., 2004), leptin (Garza et al., 2012) and insulin (Clodfelder-Miller et al., 2005). Serotonin activates Akt and deactivates GSK3β by phosphorylation in rat hippocampal neuron cultures in a PI3K-dependent manner (Chen et al., 2007; Cowen et al., 2005). Moreover, administration of dexfenfluramine resulted in analogous effects in the hippocampus of mice (Li et al., 2004; Polter et al., 2009). However, the anatomical distribution of this activation in the different regions of hippocampus has not been demonstrated yet. Only one recent study by Polter and colleagues presented the phosphorylated Akt immunoreactivity, in the hippocampus of mice, after i.p. injection with the 5-HT$_{1A}$ agonist 8-OH-DPAT but no quantification was available in the article. In the same article, levels of phosphorylated GSK3β were also displayed and it was reported that immunoreactivity was increased significantly in the CA3 and DG regions (Polter et al., 2012). In another study, it was reported that i.p. administration of fluoxetine induced an increase of pGSK3β immunostaining in hilar cells of the DG (and CA3 pyramidal cells which are not shown) but without any quantification given (Li et al., 2007). In both articles, the lack of good magnification and quantification respectively, suggested that new experiments need to be done in order to clarify this effect and its localization. Similarly, regarding leptin and insulin, there are no studies to date that describe quantified changes in the levels of phosphorylation of Akt and/or GSK3β. Elmquist and colleagues in a neuroanatomical study detected immunoreactivity of LepRb only in the granular area of the DG and in no other hippocampal area. In addition, leptin failed to activate the JAK/STAT pathway and it was suggested that the effects of leptin in this area should be mediated through another pathway such as the PI3K/Akt pathway (Scott et al., 2009). In our study, we clearly demonstrated that the

effects of these three hormones on pGSK3β levels occur primarily in neuronal cells of the subgranular layer of the DG. pAkt immunoreactivity was more scattered, intense and less concentrated in the cell bodies, so we measured the total optical density of the same region. Finally, we observed that in the DG, the area with the most elevated numbers of pGSK3β was the superior arm suggesting that this area is of great importance for the initiation of antidepressant action. Concluding, this is the first study wherein the 5-HT-, insulin-, leptin-induced immunoreactivity of phosphorylated Akt and GSK3β is demonstrated so plainly in the rodent hippocampus.

In order to better characterize the action of 5-HT, insulin and leptin in the hippocampus, we used in this study an *ex vivo* approach on brain slices. This technique allowed us to compare the individual sensitivities to each neural modulator and to assess any possible correlation/s with physiological and behavioral parameters. Moreover, it gives us the facilitation of using each animal as its own control and thus wipes out all the variability noise induced by the individuality of each animal. In parallel, it allows us to reduce the absolute number of experimental animals, assigning it as a more ethologically correct method. In addition, the use of brain slices gives the advantage of avoiding indirect actions of the administrated substance via endocrine, paracrine or polysynaptic intermediates.

The fact that cellular insulin resistance and/or leptin resistance induce reciprocally decreased sensitivity of these two hormones has been suggested to occur in the brain too in our laboratory (Benomar et al., 2005a; Benomar et al., 2005b). Similarly, as previously mentioned, we have reported the existence of a cross-talk between insulin and serotonin the hypothalamus and in neuronal cells (Papazoglou et al., 2012). Here, we also demonstrated that in the subgranular zone of the DG, insulin and leptin resistance impairs the activation of the PI3K/Akt/GSK3β pathway by insulin and leptin as expected, but also by serotonin, suggesting a cross-talk among all the three of them. This cross-talk could take place in calretinin-positive cells, as suggested by a double immunohistochemical detection of the calcium binding protein and pAkt (Annex 2). The

activation of this pathway by 5-HT in the DG may be initiated by the receptor 5-HT$_{1A}$. Indeed, it has been clearly demonstrated, with the use of SSRIs and antagonists or agonists of 5-HT$_{1A}$ receptor, that serotonin GSK3β inhibition in the hippocampus occurs after stimulation of postsynaptic 5-HT$_{1A}$ (Li et al., 2004; Polter et al., 2012). In addition, 5-HT$_{1A}$-mediated inhibition of GSK3β in the hippocampus is blocked by LY294000, indicating that this action of serotonin requires the activation of PI3K/Akt pathway (Polter et al., 2012). Furthermore, the possibility that the 5-HT-induced phosphorylation of Akt in the subgranular neurons depends on PI3K activation and involves 5-HT$_{1A}$ receptor is strengthened by preliminary experiments showing that 5-HT-dependent phosphorylation of Akt was prevented by a pre-treatment with LY294000, a PI3K inhibitor (Annex 3A-C), or NAD 299, a 5-HT$_{1A}$ antagonist (Annex 3AB,D). However, these data have to be confirmed in future experiments.

High-fat diet induces a depressive-like behavior in adult male Wistar rats

One of our major findings was that high-fat diet consumption by rats not only results in obesity and its parallel metabolic comorbidities, but also generates mood disturbances. This has been already demonstrated in mice by two studies (Sharma and Fulton, 2012; Yamada et al., 2011). Yamada *et al.* suggested that leptin exerts antidepressant action via the stimulation of BDNF expression in the hippocampus, which is impaired by a HF diet (Yamada et al., 2011). Besides, Sharma and Fulton demonstrated that HF diet alters the expression of several molecules (BDNF, D1R, D2R, ΔFos, phospho-CREB) in the reward circuit including nucleus accumbens, dorsolateral striatum and ventral tegmental area (Sharma and Fulton, 2012). Here we show that a direct effect of the HF diet on the neuronal sensitivity to 5-HT, the main neuromodulator of mood, may underlie the causal link between metabolic syndrome and depression. More precisely, our results indicate that the resistance to leptin and/or insulin induced by a HF diet alters 5-HT signaling in the subgranular neurons of the dentate gyrus. These neurons are of particular interest because they can derive from adult neurogenesis, which is required for antidepressants' action (Santarelli et al., 2003). Interestingly, by detecting

immunohistochemically Ki-67, a cell proliferation marker, we found that the HF diet decreased the cell proliferation rate in the subgranular zone, and that this effect was partially reversed after 6 weeks of food substitution (Annexes 4-5). Furthermore, we noticed that the number of Ki-67-positive cells in the subgranular zone negatively correlated with the body weight of animals. Taken together, these data reinforce the link between metabolic syndrome and adult neurogenesis in our model.

It is interesting that the nature of nutrition received by an animal can affect its psychological state in such a short time. The toxicity of a hypercaloric diet is beyond the metabolic defects that were provoking our interest until recently and reaches the spectrum of psychological disorders. This problem arises also from the fact that obesity is an obvious effect of this diet whereas the depressive–like symptoms are not that noticeable and a battery of behavioral test is needed in order to reveal such evidences. Likewise, in human beings, obesity and depression are powerfully linked according to recent epidemiological and clinical studies (Faith et al., 2011). Equally noteworthy is the finding that diet reversion restores not only food intake but also behavioral aspects such as depressive-like behavior before reaching the body weight and the glycemia of control animals. Moreover, sensitivity to insulin, leptin and serotonin was reestablished too in the dentate gyrus of HF/C rats. This is in accordance with a recent study showing that diet reversion restores insulin sensitivity and transport in rat brain (Begg et al., 2013). Thus, these data suggest that food quality is an important factor not only for the introduction of metabolic syndrome but also for the rescue from these effects.

CONCLUSION

Conclusion

In this thesis, we aimed to investigate the consequences of insulin-resistance states on the serotonin signaling in the brain. We hypothesized that the PI3K/Akt signaling pathway could be shared by both serotonin and insulin in several regions, including the hypothalamus, a key center of the control of energy balance, and the hippocampus, which is widely involved in the mood regulation. Indeed, these regions express high levels of insulin and serotonin receptors. To address this question, we used two rat models of insulin resistance, the Goto-Kakizaki rats, which exhibit spontaneous type 2 diabetes, and a diet-induced obesity model, which is characterized by joint insulin- and leptin-resistance. Moreover, we mimicked insulin-resistance in a human neuroblastoma cell line (the SH-SY5Y cells) to further dissect the interactions between insulin and serotonin signaling. Interestingly, we evidenced a serotonin-dependent activation of the PI3K/Akt pathway in the hypothalamus, the hippocampus and the neuroblastoma cells, which was altered in insulin resistant models. These observations suggest the existence of a cross-talk between serotonin and insulin in these brain regions, which could putatively occur in other areas of the central nervous system too.

Consequently, an impairment of the serotonergic signaling in the hypothalamus due to insulin resistance can exacerbate a number of dysfunctions in the control of energy homeostasis. Moreover, hypothalamic impairment of the PI3K/Akt pathway can signify many modifications to downstream signaling cascades and result in some of the symptoms of type 2 diabetes. Thus, we propose that serotonin-induced Akt activation in the various hypothalamic nuclei should be investigated as well as the physiological outcomes of this action in different neuronal subgroups. Furthermore, the putative serotonin receptors that are responsible for the initiation of the PI3K/Akt pathway stimulation in the hypothalamus should be tested. The modulation of other metabolism-related signaling pathways such as the JAK2/STAT3 pathway should be investigated in this region. Finally, a study of downstream targets of Akt, such as FOXO1 and GSK3β,

which are key players in the regulation of energy balance, would provide a better understanding of the effects of serotonin.

Likewise, insulin resistance in the high-fat diet model decreased activation of Akt and increased the activation of the Akt substrate GSK3β by serotonin in the hippocampus. In parallel, this model displayed depressive-like behavior, associated to impaired inhibition of GSK3β by serotonin in the dentate gyrus. This observation allows us to conclude that the alteration of serotonin signaling in insulin-resistant states accounts for the depressive behavior evidenced in these animals. Thus, the cross-talk between insulin and serotonin signaling could represent a neurobiological mechanism underlying the association between metabolic syndrome and depression. In addition, the alteration of the antidepressant serotoninergic effects in diabetic and/or obese models should be tested in order to confirm the causal relationship between insulin-resistance and depression. Finally, the complete mechanism of this cross-talk remains to be further clarified. The type of 5-HT receptor through which PI3K/Akt/GSK3β is stimulated needs to be investigated in future studies. In addition, the cellular components that are involved in the interaction between serotonin and insulin signaling should be characterized.

Taken the information provided by this thesis all together, we can conclude that a cross-talk between serotonin and insulin (and leptin in the DG) signaling pathways exists in the hypothalamus and the hippocampus (Figure 16).
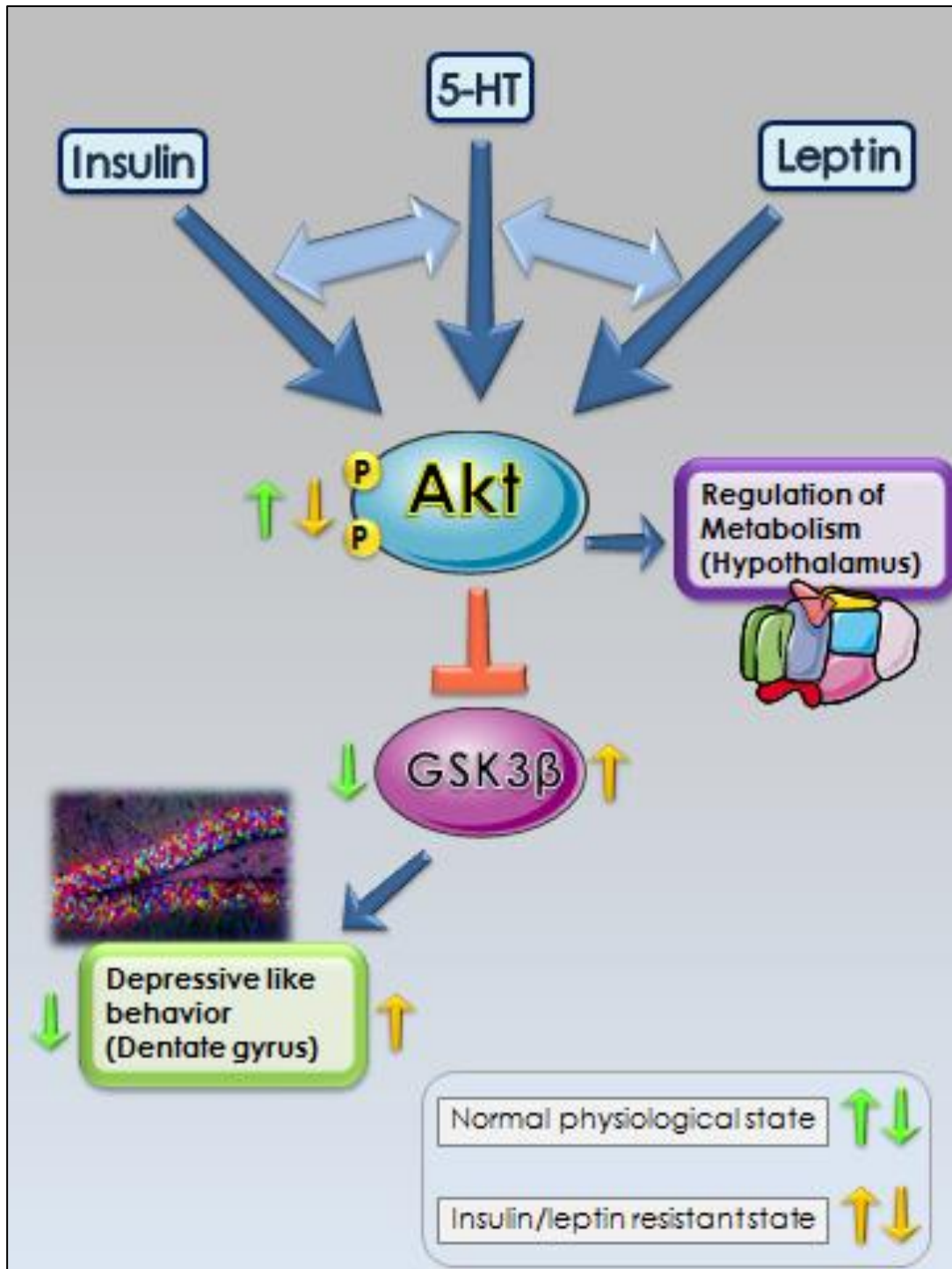
Figure 16: Cross-talk between serotonin, insulin and leptin in the brain through the PI3K/Akt/GSK3β signaling pathway.

ANNEXES

Annex 1

"Extracellular progranulin protects cortical neurons from toxic insults by activating survival signaling."
Neurobiol Aging. 32(12):2326.e5-16.

Xu J., Xilouri M., Bruban J., Shioi J., Shao Z., Papazoglou I., Vekrellis K., Robakis N.K. (2011).

Extracellular progranulin protects cortical neurons from toxic insults by activating survival signaling

Jindong Xu[a,1,2], Maria Xilouri[b,2], Julien Bruban[a], Junichi Shioi[a], Zhiping Shao[a],
Ioannis Papazoglou[b,c], Kostas Vekrellis[b], Nikolaos K. Robakis[a,*]

[a] Center for Molecular Biology and Genetics of Neurodegeneration, Departments of Psychiatry and Neuroscience, Mount Sinai School of Medicine, New York, NY, USA
[b] Division of Basic Neurosciences, Biomedical Research Foundation of the Academy of Athens, Athens, Greece
[c] Neuroendocrinologie Moléculaire de la Prise Alimentaire, University of Paris-Sud, UMR 8195, Orsay, France

Abstract

To reduce damage from toxic insults such as glutamate excitotoxicity and oxidative stresses, neurons may deploy an array of neuroprotective mechanisms. Recent reports show that progranulin (*PGRN*) gene null or missense mutations leading to inactive protein, are linked to frontotemporal lobar degeneration (FTLD), suggesting that survival of certain neuronal populations needs full expression of functional PGRN. Here we show that extracellular PGRN stimulates phosphorylation/activation of the neuronal MEK/extracellular regulated kinase (ERK)/p90 ribosomal S6 kinase (p90RSK) and phosphatidylinositol-3 kinase (PI3K)/Akt cell survival pathways and rescues cortical neurons from cell death induced by glutamate or oxidative stress. Pharmacological inhibition of MEK/ERK/p90RSK signaling blocks the PGRN-induced phosphorylation and neuroprotection against glutamate toxicity while inhibition of either MEK/ERK/p90RSK or PI3K/Akt blocks PGRN protection against neurotoxin MPP$^+$. Inhibition of both pathways had synergistic effects on PGRN-dependent neuroprotection against MPP$^+$ toxicity suggesting both pathways contribute to the neuroprotective activities of PGRN. Extracellular PGRN is remarkably stable in neuronal cultures indicating neuroprotective activities are associated with full-length protein. Together, our data show that extracellular PGRN acts as a neuroprotective factor and support the hypothesis that in FTLD reduction of functional brain PGRN results in reduced survival signaling and decreased neuronal protection against excitotoxicity and oxidative stress leading to accelerated neuronal cell death. That extracellular PGRN has neuroprotective functions against toxic insults suggests that in vitro preparations of this protein may be used therapeutically.
© 2011 Elsevier Inc. All rights reserved.

Keywords: Progranulin; Neuroprotection; Neurodegeneration; Frontotemporal lobar degeneration; Excitotoxicity; Oxidative stress; ERK; Akt

Abbreviations: BDNF: brain-derived neurotrophic factor; BSA: bovine serum albumin; DMEM: Dulbecco's modified Eagle's medium; EDTA: ethylenediaminetetraacetic acid; ERK: extracellular signal-regulated kinase; FBS: fetal bovine serum; MEK: MAPK/ERK kinases; MPP$^+$: 1-methyl-4-phenylpyridinium; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MTT: methylthiazolyldiphenyl-tetrazolium bromide; p-: phosphorylated-; PAGE: polyacrylamide gel electrophoresis; PBS: phosphate buffer saline; PC: pheochromocytoma; PCDGF: S6 kinase; SDS: sodium dodecyl sulfate; TAR: trans-activation response element; LDH: lactate dehydrogenase.

1. Introduction

Progranulin (PGRN), also identified in literature as PC cell-derived growth factor (PCDGF), acrogranin, or proepithelin, is a 593 residue polypeptide that contains a signal sequence and 7.5 homologous cysteine-rich granulin domains separated by linker sequences (Bateman and Bennett, 2009). Mature PGRN is secreted as a highly glycosylated

---

* Corresponding author at: Center for Molecular Biology and Genetics of Neurodegeneration, Departments of Psychiatry and Neuroscience, Mount Sinai School of Medicine, One Gustave Levy Place, Box 1229, New York, NY 10029, USA. Tel.: +1 212 241 9380; fax: +1 212 241 0760.

E-mail address: nikos.robakis@mssm.edu (N.K. Robakis).

[1] Current address: McKnight Brain Institute and Department of Neurology, University of Florida College of Medicine, 100 S. Newell Drive, Gainesville, FL 32611, USA.

[2] These authors contributed equally to this work.

protein of approximately 88–95 kDa that under certain conditions is processed in the linker regions by elastase or other proteases to produce biologically active peptides referred to as granulins or epithelins (Butler et al., 2008; Kessenbrock et al., 2008; Zhu et al., 2002). Processing of secreted PGRN is inhibited by the secretory leukocyte protease inhibitor (SLPI) (Zhu et al., 2002). PGRN and its derivatives, granulin peptides, are expressed in many tissues including epithelial cells, the gastrointestinal tract and hematopoietic cells (Daniel et al., 2000) and have been associated with multiple biological functions such as regulation of cell growth, cell cycle progression, embryonic development, and tissue repair (Bateman and Bennett, 2009; He and Bateman, 2003). Studies in nonneuronal systems indicate that PGRN activates cell signaling pathways including the extracellular regulated kinase (ERK1/2) and the phosphatidylinositol-3 kinase (PI3K)/Akt cell survival pathways (He et al., 2002; Lu and Serrero, 2001; Monami et al., 2006; Zanocco-Marani et al., 1999). During inflammation and wound healing, cells secrete the protease elastase that converts PGRN to granulin peptides which may have different, overlapping, or even opposite functions from the parent protein (Plowman et al., 1992; Tolkatchev et al., 2008; Zhu et al., 2002). For example, while full length PGRN acts as an anti-inflammatory agent, individual granulin peptides have been shown to stimulate production of proinflammatory cytokines (Zhu et al., 2002). These observations suggest that a carefully maintained equilibrium between PGRN and granulin peptides may be important to tissue homeostasis.

In the central nervous system (CNS), PGRN is expressed in both neurons and microglia (Daniel et al., 2000; Matsuwaki et al., 2011; Ryan et al., 2009). Importantly, recent genetic studies showed that PGRN mutations are linked to frontotemporal lobar degeneration (FTLD), a form of dementia characterized by severe neuronal loss in the frontal and temporal brain regions of adult patients (Baker et al., 2006; Cruts et al., 2006). These findings renewed interest in the brain functions of this protein. To date, more than 70 FTLD-linked *PGRN* mutations have been detected with most of them causing functional null alleles (Sleegers et al., 2010). Although the autosomal dominant mode of inheritance of PGRN-linked familial FTLD might suggest gain of a toxic function, many *PGRN* mutants encode incomplete or inactive peptides indicating that PGRN haploinsufficiency can result in dominant transmission of neurodegeneration. Based on these findings, decreased plasma PGRN levels has been proposed as a biomarker for early diagnosis of this disorder (Finch et al., 2009; Ghidoni et al., 2008; Sleegers et al., 2009). Besides the null FTLD mutations that lead to decreased PGRN levels, there are at least 17 PGRN missense mutations linked to FTLD families (Gijselinck et al., 2008). The pathogenetic nature of these mutations is less clear than the PGRN null mutations, but it was recently reported that at least some missense mutations also cause a decrease in the levels of functional PGRN (Shankaran et al., 2008; Wang et al., 2010). Thus, there is strong evidence that a 50% reduction of functional PGRN (haploinsufficiency) leads to increased neuronal cell death in adult brains supporting the hypothesis that PGRN or its derivatives promote neuronal survival.

Recently, it was reported that secreted PGRN binds sortilin and is subsequently delivered to lysosome through endocytosis (Hu et al., 2010). Additional studies suggest that PGRN functions as a neurotrophic agent that may promote survival of primary neuronal cultures under conditions of serum withdrawal (Gao et al., 2010; Ryan et al., 2009; Van Damme et al., 2008). Furthermore, PGRN knockdown decreases axonal outgrowth in zebra fish embryos and PGRN-deficient hippocampal slices are susceptible to glucose deprivation (Laird et al., 2010; Yin et al., 2010). Together, these reports indicate that PGRN functions as a neurotrophic factor and may play important roles in neuronal physiology.

That reduction of PGRN leads to increased degeneration of cortical neurons in the CNS (Bateman and Bennett, 2009; Sleegers et al., 2010), raises the possibility this protein functions as a neuroprotective factor. For example, PGRN may protect brain neurons from exposure to toxic insults such as glutamate-associated excitotoxicity and oxidative stress. Both processes are associated with neuronal activity and have been proposed to play important roles in the development neurodegenerative disorders (Fatokun et al., 2008; Lau and Tymianski, 2010). Here we show that extracellular PGRN activates neuronal ERK1/2 and Akt cell survival signaling and protects neurons from toxic insults associated with neurodegeneration.

2. Methods

All chemicals were purchased from Sigma, (St. Louis, MO, USA) except where indicated. All animal experiments were carried out in accordance with the rules and regulations at the Mount Sinai School of Medicine and the Biomedical Research Foundation of the Academy of Athens.

2.1. Primary neuronal cultures

Cortical neuronal cultures from embryonic brains of E18.5 Wistar rats were prepared as described (Vogiatzi et al., 2008; Xu et al., 2009). Dissociated cells were plated onto poly-D-lysine-coated plates at a density of approximately 60,000/cm^2. Cells were maintained in Neurobasal medium supplemented with 1% B27 (Invitrogen, Eugene, OR, USA), L-glutamine (0.5 mM) and penicillin/streptomycin (1% vol/vol) and used at 8 to 12 days in vitro (DIV). Under these conditions postmitotic neurons represent more than 98% of cultured cells (Paxinou et al., 2001).

2.2. Production and purification of PGRN

Human embryonic kidney (HEK293)T cells stably transfected with vector pcDNA3.1/V5-His-TOPO expressing human PGRN (provided by Dr. Bateman, McGill University) were grown in DMEM supplemented with 10% FBS and 0.5 mg/mL G418. At 100% cell confluency, the growth medium was replaced with fresh DMEM and conditioned media were collected 48 hours later. An approximately 90-kDa protein corresponding to glycosylated full-length PGRN was present in conditioned media of transfected, but not control, cultures (see Supplementary Fig. 1). His-tagged PGRN was bound to Ni-NTA agarose beads (Qiagen, Valencia, CA, USA) overnight and collected beads were washed with PBS containing 10 mM imidazole. PGRN was then eluted with PBS containing 200 mM imidazole. Silver-stained SDS gels showed purified full-length PGRN protein with minimal degradation (Supplementary Fig. 1). For PGRN stability experiments, PGRN was added to neuronal cultures for various times as indicated in the text. Elastase alone (0.3 U/mL) or elastase preincubated with protease inhibitors for 30 minutes, was added to the media together with PGRN. At the end of reaction, media were retrieved, denatured and analyzed by Western blot using anti-V5 tag antibody (Invitrogen).

2.3. Cell survival assays

Three independent assays were used to measure PGRN-dependent neuroprotection against glutamate or H_2O_2 toxicity as indicated in Results. Hoechst staining assay of neuronal viability (Arndt-Jovin and Jovin, 1977) was determined according to manufacturer's instructions (Sigma). Briefly, neurons on poly-D-lysine-coated 24-well plates were treated with glutamate for 3 hours, fixed in 4% paraformaldehyde for 20 minutes at room temperature and stained with Hoechst 33342 for 10 minutes. Neurons were then observed under a fluorescence microscope on ultraviolet illumination. Numbers of viable neurons were counted in 10 fields per well with at least 20 neurons per field. Results are expressed as percent of control value. MTT cell viability assay based on the cleavage of yellow tetrazolium salt MTT to purple formazan (Denizot and Lang, 1986) was performed according to manufacturer's instructions (Sigma). In summary, neurons at 8–12 DIV grown on poly-D-lysine-coated 96-well plates were treated with toxic agents for 3 hours and MTT solution (1 mg/mL) was added to each well. Plates were incubated at 37 °C for 2 hours and the reaction was terminated by 0.1 N HCl in isopropanol for 1 hour. Absorbance was then measured at 560 nm by spectrophotometric microplate reader (Thermo Scientific, Hudson, NH, USA) with background subtraction at 620 nm. Data are expressed as a percentage of control value. Lactate dehydrogenase (LDH) release assays (Koh and Choi, 1987), were performed using the cytotoxicity Detection Kit plus (Roche, New Jersey, NJ, USA) according to manufacturer's

instruction. Results are expressed as the percentage of LDH release by nontreated cells.

2.4. Assessment of MPP^+ toxicity

Rat cortical neurons cultured on poly-D-lysine-coated 12-well plates for 5 days were treated with 35 nM progranulin or bovine serum albumin (BSA) (as control) for 24 hours prior to addition of 40 μM 1-methyl-4-phenylpyridinium (MPP^+). Twenty-four hours after MPP^+ addition, culture media were removed and cells were lysed in a detergent containing solution that enables the quantification of viable cells by counting the number of intact nuclei in a hemacytometer as described (Farinelli et al., 1998; Stefanis et al., 1999). Cell counts were performed in triplicate and are reported as mean ± standard error (SE). In experiments with inhibitors of ERK and Akt MPP^+ treatment was modified in order to achieve comparable death rates in shorter time periods. This was crucial because inhibitors are unstable in culture and may exert nonspecific toxic effects when used for longer times. Titration experiments with or without the inhibitors showed that 4-hour treatments with the compounds was the optimal regimen. Axonal degeneration and loss of neuritic processes were observed using phase-contrast microscopy.

2.5. Western blot analysis

Western blot analysis was performed as described (Vogiatzi et al., 2008; Xu et al., 2009). Briefly, neurons were washed with cold PBS and solubilized in lysis buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, phosphatase and protease inhibitor cocktail. Cell lysates were centrifuged at 16,000*g* for 40 minutes and 30 μg of supernatant protein was loaded onto each well of 10% SDS-PAGE. The following antibodies were used for blotting analysis: anti-PGRN (Zymed, San Francisco, CA, USA), anti-His (Qiagen), anti-V5 (Invitrogen), anti-ERK1/2 and pERK Thr202/Tyr204 (Cell Signaling, Beverly, MA, USA), anti-Akt and pAkt Ser473 (Cell Signaling), anti-p90RSK and pRSK Thr359/Ser363 as well as pRSK Ser380 (Cell Signaling).

2.6. Statistical analysis

All data are expressed as mean ± SE. All data were normalized to the control (100%) and Levene's test (embedded in SPSS [IBM SPSS statistics, version 19]) was used to assess the homogeneity of variance. Accordingly, statistical significance of differences was evaluated either with paired *t* test or with one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls' test as post hoc multiple comparisons depending on the significance of Levene's test. *p* values < 0.05 were considered significant.

3. Results

3.1. Extracellular PGRN promotes neuronal survival against toxic insults

Decreased expression of PGRN is associated with increased degeneration of cortical neurons in the CNS (Bateman and Bennett, 2009; Sleegers et al., 2010), raising the possibility this protein functions as a neuroprotective factor. For example, PGRN may protect brain neuronal populations from exposure to toxic insults such as glutamate-associated excitotoxity and oxidative stress. To better understand the effects of PGRN on neuronal survival, we generated adequate amounts of purified protein using recombinant DNA technology (see Methods) and asked whether exogenous PGRN is able to protect rat cortical neuronal cultures from glutamate toxicity. Treatment of our cultures with 50 μM glutamate reduced neuronal cell viability to 53% $\pm$ 4%, determined by Hoechst staining, a commonly used protocol that evaluates cell survival by counting intact cell nuclei (see Methods). Pretreatment of the neuronal cultures with recombinant PGRN however, significantly decreased the glutamate-induced neuronal cell death (73% $\pm$ 5% survival) (Fig. 1A). The neuroprotective effect of PGRN against glutamate excitotoxicity was verified by employing the MTT as well as the LDH assays, both of which are commonly used to evaluate cell toxicity and survival (Fig. 1B and 1C, respectively). Glutamate toxicity, measured by the amount of LDH released to the culture medium, was reduced by PGRN proportionally to its concentration and the effect of PGRN at 35 nM was comparable to the neuroprotective effect induced by brain-derived neurotrophic factor (BDNF) used as a positive control (Fig. 1C).

To explore whether PGRN is able to protect neurons from oxidative stress, we treated rat cortical neuronal cultures with hydrogen peroxide (H_2O_2), a commonly used reagent for induction of oxidative stress. Addition of 25 μM H_2O_2 reduced neuronal viability, measured using the MTT assay, to about 46% $\pm$ 4% of control while preincubation with 35 nM PGRN significantly protected neurons from oxidative damage increasing cell survival to approximately 54% $\pm$ 5% of control. BSA used at concentrations similar to PGRN had no effect on neuronal cell viability (Fig. 1D). MPP^+, the active derivative of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), is known to exert neuronal toxicity, in part, by releasing reactive oxygen species (ROS) from the mitochondria, as a result of Complex I inhibition (Przedborski and Vila, 2003). To study the PGRN effects against MPP^+ toxicity, we exposed cortical neurons to 40 μM MPP^+ for 24 hours and cell survival was evaluated by counting intact nuclei. MPP^+ treatment decreased neuronal survival to 47% $\pm$ 7% of control (Fig. 1E), while pretreatment of these cultures with 35 nM of PGRN restored survival to nearly control levels (Fig. 1E). In contrast, pretreatment with BSA had no effect on the MPP^+-induced neuronal death (Fig. 1E). Together, these results indicate

that PGRN has a strong neuroprotective effect against MPP^+ neurotoxicity. Furthermore, our data suggest that the neuroprotective effect of PGRN depends on the toxic agent. Thus, there is a significance difference between PGRN and Glu+PGRN (p = 0.016; Fig. 1A) but no significance between PGRN and MPP^+ + PGRN (p = 0.516; Fig. 1E) suggesting PGRN almost completely reverses the toxic effects of MPP^+ but not those of glutamate.

Depending on culture conditions and cell type, secreted PGRN can be processed by extracellular proteases, including elastase and proteinase 3, to produce granulin peptides that have been proposed to have different functions from the parent protein (He and Bateman, 2003; Kessenbrock et al., 2008; Zhu et al., 2002). To explore whether neurons secrete proteases able to process PGRN, purified protein was added to the medium of rat cortical neuronal cultures, and nondegraded PGRN was recovered and analyzed. Fig. 2 shows that PGRN is remarkably stable in the culture media of primary neurons even after overnight incubation (lanes 2, 3, 6, and 9). In contrast, PGRN was quickly degraded in the presence of exogenous elastase (lanes 4 and 7), a process inhibited by inhibitors of elastase (lanes 5 and 8). These data show that exogenous PGRN is not metabolized to any significant extent by primary neuronal cultures in vitro and suggest that it is unlikely that this protein is processed to granulins by cortical neurons in vivo. Furthermore, our observations suggest that the neuroprotective properties of exogenous PGRN are most probably due to full-length PGRN protein rather than granulin peptides.

3.2. Extracellular PGRN activates ERK and Akt signaling in cortical neurons

To elucidate the mechanism by which PGRN exerts its neuroprotective function, we examined its effects on cell signaling pathways including those of ERK1/2 and Akt kinases. Both pathways have been reported to be activated in response to PGRN in nonneuronal cell lines (He et al., 2002; Lu and Serrero, 2001; Monami et al., 2006; Zanocco-Marani et al., 1999) and we reasoned that these may also be activated in primary neurons. ERK1/2 and Akt kinases are involved in cell survival and activation of the corresponding signaling pathways is indicated by phosphorylation of ERK1/2 at residues Thr202/Tyr204 and of Akt at Ser473 (Fayard et al., 2005; Payne et al., 1991). Fig. 3A shows that PGRN treatment leads to a rapid increase in the phosphorylation of both ERK1/2 and Akt kinases at residues Thr202/Tyr204 and Ser473, respectively. Interestingly, ERK1/2 phosphorylation peaked earlier than Akt phosphorylation (Fig. 3A) and because there is no evidence that Akt is downstream of ERK, these PGRN-induced phosphorylation events may be independent of each other suggesting PGRN independently activates both survival pathways. To confirm the specificity of ERK and Akt activation by PGRN, we employed pharmacological agents that specifically target MEK/ERK1/2 and PI3K/Akt kinases. Preincubation of our cultures with MEK/ERK1/2 inhibitor U0126
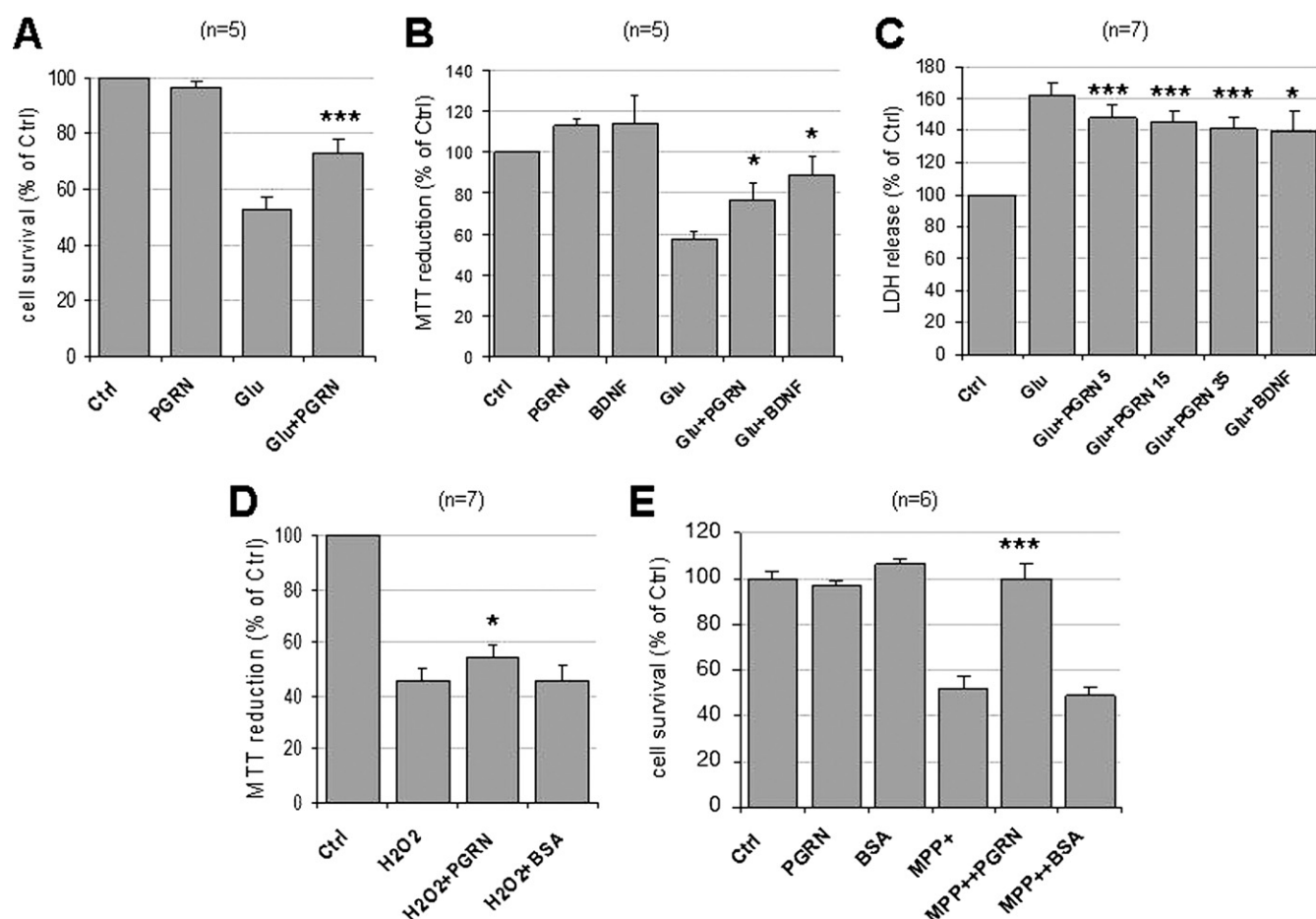
Fig. 1. Neuroprotective functions of extracellular progranulin (PGRN). (A) Rat cortical neurons were cultured in 24-well plates in neurobasal media plus B27 supplement. At 8–12 days in vitro (DIV), neurons were pretreated with 35 nM PGRN for 30 minutes followed by 50 μM glutamate incubation for 3 hours. Cells were then fixed with 4% paraformaldehyde and stained with Hoechst 33342. Five pictures were taken from each well and each condition represented the average of 4 wells. Cell survival was measured by counting the number of cells with normal nuclear morphology. Results (mean $\pm$ standard error [SE]) were calculated from 5 independent experiments. *** $p < 0.005$ comparing between cultures treated with glutamate in the presence or absence of PGRN (paired t test). (B) Cortical neurons as above cultured in 96-well plates were pretreated with 35 nM PGRN or 50 ng/mL brain-derived neurotrophic factor (BDNF) for 18 hours followed by 50 μM glutamate for 3 hours. Cell viability was evaluated by MTT assay and normalized to control (ctrl). Results (mean $\pm$ SE) were summarized from 5 independent experiments and in each experiment each condition is the average of 6 identical wells. * $p < 0.05$ comparing between cultures treated with glutamate in the presence or absence of PGRN or BDNF (paired t test). (C) Neuronal cultures as above were pretreated with different concentrations (5 nM, 15 nM, or 35 nM) of PGRN or 50 ng/mL BDNF for 2 hours followed by 50 μM of glutamate treatment for 24 hours. The cell-free culture supernatant was collected and LDH release was determined as per manufacturer's instructions. Results (mean $\pm$ SE) were summarized from 7 independent experiments. * $p < 0.05$; *** $p < 0.005$ comparing between cultures treated with glutamate in the presence and absence of PGRN or BDNF (paired t test). Numbers next to PGRN indicate concentrations in nM. (D) Rat cortical neuronal cultures as in (B) were pretreated with either 35 nM PGRN or BSA for 24 hours followed by 25 μM H_2O_2 treatment overnight. Cell viability was evaluated as in (B) and normalized to control. Results (mean $\pm$ SE) were summarized from 7 independent experiments and in each experiment each condition is the average of 6 identical wells. * $p < 0.05$ comparing glutamate-treated cultures in the presence or absence of PGRN (paired t test). (E) Neurons in 12-well plates were pretreated with either 35 nM PGRN or BSA for 24 hours followed by 40 μM MPP$^+$ treatment for 24 hours. Cell survival was evaluated by counting the number of intact nuclei in a hemacytometer after lysis (see Methods). Results (mean $\pm$ SE) were summarized from 6 independent experiments. *** $p < 0.005$ comparing between cultures treated with glutamate in the presence and absence of PGRN (paired t test). Ctrl, no treatment; Glu, glutamate.

decreased phosphorylation of ERK1/2 while pretreatment with PI3K/Akt inhibitor LY-294002 abolished PGRN-induced Akt phosphorylation. MEK/ERK1/2 inhibitor PD98059 and PI3K/Akt inhibitor wortmannin gave results similar to those obtained with U0126 and LY-294002 respectively (Fig. 3B and C).

An important downstream effector of ERK1/2 kinase is the p90 ribosomal S6 kinase (p90RSK) which is activated upon phosphorylation at residues Thr359/Ser363 and

Ser380. Following activation p90RSK travels to the nucleus where it regulates gene expression (Anjum and Blenis, 2008). Fig. 4 shows that treatment of neuronal cultures with PGRN induced a rapid increase in the phosphorylation of p90RSK residues Thr359/Ser363 and Ser380. Furthermore, the PGRN-induced phosphorylation of p90RSK was blocked by MEK/ERK1/2 inhibitor U0126 indicating that ERK1/2 mediates the PGRN-induced phosphorylation of
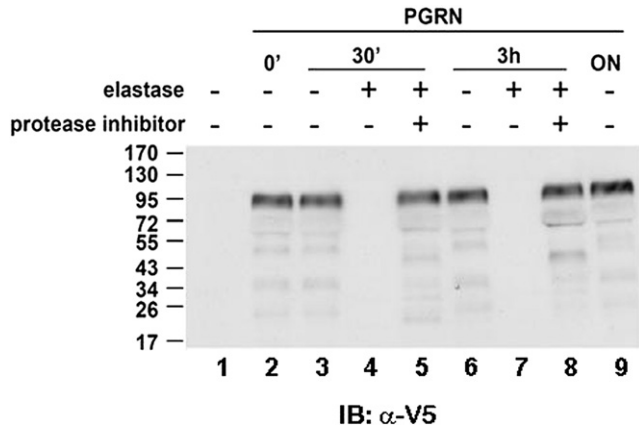
Fig. 2. Stability of extracellular PGRN in neuronal culture media. Thirty-five nM of recombinant PGRN (see Methods) was added to neuronal cultures for various time periods as indicated. Elastase (0.3 U/mL) or mixture of elastase with protease inhibitor cocktail was added together with PGRN at the same time. Protease inhibitors were preincubated with elastase for 30 minutes before adding into culture media. At the end of reaction, 5 μL of media were retrieved, denatured and analyzed by Western blot with anti-V5 tag antibody. Numbers to the left of the blots indicate the position and size (kDa) of molecular mass markers. IB, immunoblot.

p90RSK. Taken together, these results show that PGRN specifically activates the ERK1/2 and Akt cell survival pathways in neuronal populations and suggest a mechanism by which PGRN protects neurons from toxic insults.

3.3. Inhibition of ERK1/2 and Akt signaling abolishes the neuroprotective effects of extracellular PGRN

To examine whether the PGRN-dependent neuroprotection is indeed mediated by ERK1/2 signaling, we used MEK/ERK1/2 inhibitor U0126. Fig. 5A shows that the PGRN-dependent neuronal survival of glutamate-treated cultures is inhibited by U0126. Consistent with these results obtained with the cell counting assay, U0126 also blocked the PGRN-dependent decrease of LDH in the media of glutamate-treated neuronal cultures (Fig. 5B). U0126 alone had no effect on cell survival or LDH release (Fig. 5). Despite the PGRN-induced increase in Akt phosphorylation however, Akt inhibitors, including LY-294002, had no effect on the PGRN-dependent neuroprotection (Supplementary Fig. 2), suggesting that the Akt signaling has little or no effect on the neuroprotective activities of PGRN against glutamate toxicity and that MEK/ERK1/2 signaling may be the main pathway mediating the PGRN neuroprotection against glutamate excitotoxicity.

We then employed pharmacological inhibitors to ask whether the ERK1/2 and Akt signaling pathways are involved in the neuroprotective function of PGRN against MPP$^+$-induced toxicity. Fig. 6A shows that PGRN significantly inhibited the neuronal cell death induced by toxin MPP$^+$ and this neuroprotective effect was blocked by MEK/ERK1/2 inhibitors including U0126 and PD98059. In contrast to the glutamate-induced tox-

icity however, the neuroprotective effect of PGRN against MPP$^+$ toxicity was partially blocked by inhibitors of the PI3K/Akt cell survival signaling like LY-294002 and wortmannin (Fig. 6A). These data suggest that both the ERK1/2 and Akt signaling pathways contribute to the neuroprotective activities of PGRN against MPP$^+$ toxicity. This suggestion is further supported by data that inhibition of both MEK/ERK1/2 and PI3K/Akt pathways act synergistically suppressing further the PGRN-dependent neuronal survival of MPP$^+$-treated cultures (Fig. 6A). Furthermore, our data indicate that these pathways contribute independently to the neuroprotective effects of PGRN against MPP$^+$ toxicity. Morphological examination of MPP$^+$-treated neuronal cultures showed that PGRN decreases degeneration of neuritic processes caused by exposure to MPP$^+$ and this protective process is blocked in the presence of inhibitors against both MEK/ERK1/2 and PI3K/Akt kinases (Fig. 6B). Together, our results reveal a novel neuroprotective function of PGRN against toxic agents, such as neurotoxin MPP$^+$, and suggest that this neuroprotective function of PGRN involves activation of both ERK1/2 and Akt cell survival signaling pathways.

4. Discussion

Progranulin is a secreted protein that has been shown to play important roles in many biological processes including inflammation, wound repair, tumorgenesis, and embryonic development (Bateman and Bennett, 2009). Genetic studies show that PGRN mutations leading to reduced levels of functional protein (haploinsufficiency) associate with specific neurodegeneration in the frontotemporal region of the brain (Baker et al., 2006; Cruts et al., 2006) suggesting that PGRN functions in neuronal physiology and survival, and that specific cortical neuronal populations may need full protein expression for sustained survival. Indeed, recent studies indicate that PGRN has neurotrophic activities and promotes neuronal survival under conditions of serum or trophic factor withdrawal (Gao et al., 2010; Ryan et al., 2009; Van Damme et al., 2008). Chronic exposure of brain neurons to toxic insults such as glutamate excitotoxicity and oxidative stress however, has been proposed as an important factor contributing to neurodegenerative disorders characterized by progressive loss of cortical neurons (Fatokun et al., 2008; Lau and Tymianski, 2010). In addition, these neurotoxic mechanisms may operate in acute conditions like stroke where production of oxygen free radicals or hyperactivity of glutamate receptors may compound neuronal damage and death. We reason that to avoid or minimize severe neuronal damage inflicted by toxic insults, neurons may deploy an array of neuroprotective mechanisms and that PGRN may be part of this neuronal defense against toxic insults. Neurons deprived of the full protection of PGRN, like in FTLD-linked PGRN mutations, may then be more vulnerable to toxic conditions than neurons that express normal level of PGRN. Over the years this chronic
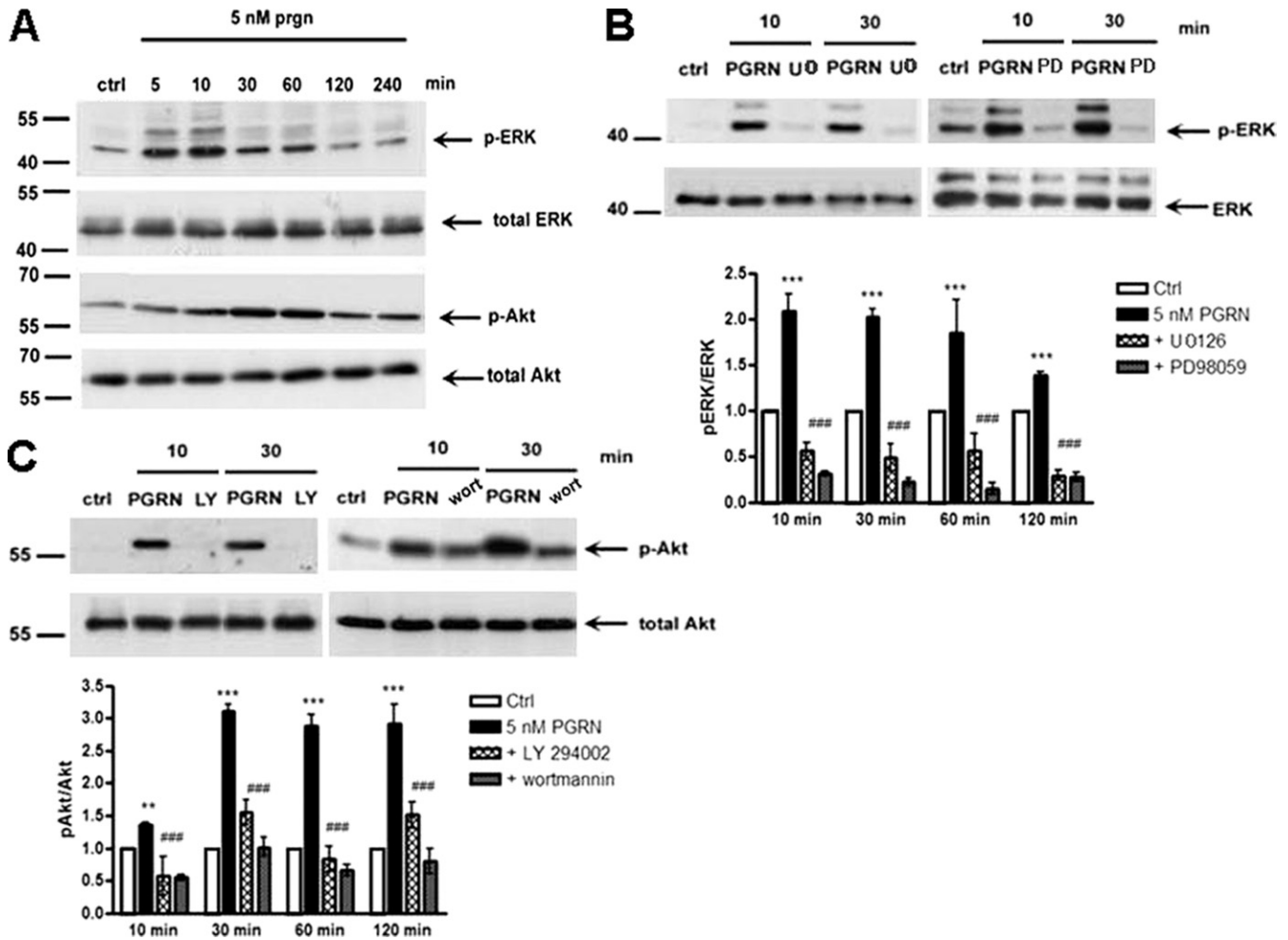
Fig. 3. Extracellular PGRN activates extracellular regulated kinase (ERK) and Akt signaling pathways of cortical neurons. (A) Rat cortical neurons in 12-well plates were treated at 8 DIV with 5 nM PGRN for the indicated time periods. Untreated cultures were used as controls (ctrl). Following incubation, cells were collected and assayed on Western blots for the proteins indicated to the right of the blots. A representative blot out of 3 independent experiments is shown. (B) ERK inhibitors U0126 (U0) and PD98059 (PD; 25 μM each) blocked PGRN-induced ERK1/2 phosphorylation. Inhibitors were added to cultures for 30 minutes prior to addition of 5 nM PGRN. Neurons were subsequently collected at indicated times and subjected to SDS-PAGE and Western blot as above. Densitometric analysis of the amounts of p-ERK in the presence of inhibitors expressed as phospho-ERK (p-ERK) to ERK ratio that was set as 1 for control (white bar). Other bars represent phosphoprotein to protein ratios relative to control. (C) Phosphatidylinositol-3 kinase (PI3K)/Akt inhibitors wortmannin (wort; 1 μM) or LY-294002 (LY; 25 μM) blocks PGRN-induced Akt phosphorylation. Inhibitors and PGRN were added to cultures as in (B) and neurons were collected at indicated times and subjected to SDS-PAGE and Western blot as above. Densitometric analysis of the amounts of p-Akt in the presence of inhibitors is expressed as p-Akt to Akt ratio as above. Data were obtained from 3 separate experiments (** $p < 0.01$, *** $p < 0.001$, one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls' test, comparing between cultures treated with PGRN and without treatment (ctrl); ### $p < 0.001$, comparing between cultures treated with PGRN in the presence or absence of inhibitors. Signal variability is indicated by error bars.

vulnerability may translate into accelerated neuronal cell loss and dementia.

PGRN is secreted as a glycosylated polypeptide indicating that it exerts at least some of its biological functions in the extracellular space. To explore the neuroprotective properties of extracellular PGRN and to preserve possible modifications specific to mammalian cells, we produced and purified PGRN protein from the culture media of human embryonic kidney (HEK293) cells overexpressing human PGRN. Purified protein was then tested in primary neuronal cultures to ask whether PGRN protects neurons from specific toxic insults such as glutamate-associated excitotoxic-

ity and oxidative stresses evoked by H_2O_2 and MPP^+. Our results show that extracellular PGRN has potent neuroprotective functions mediated, at least in part, by the activation of neuronal MEK/ERK and PI3K/Akt signal transduction pathways both of which are stimulated by extracellular PGRN.

Under certain conditions including inflammation, PGRN is processed by specific proteases, such as elastase, to granulin peptides proposed to mediate some of the functions of the parent protein (Plowman et al., 1992; Tolkatchev et al., 2008; Zhu et al., 2002). We thus examined the extent to which extracellular PGRN is processed in the medium of
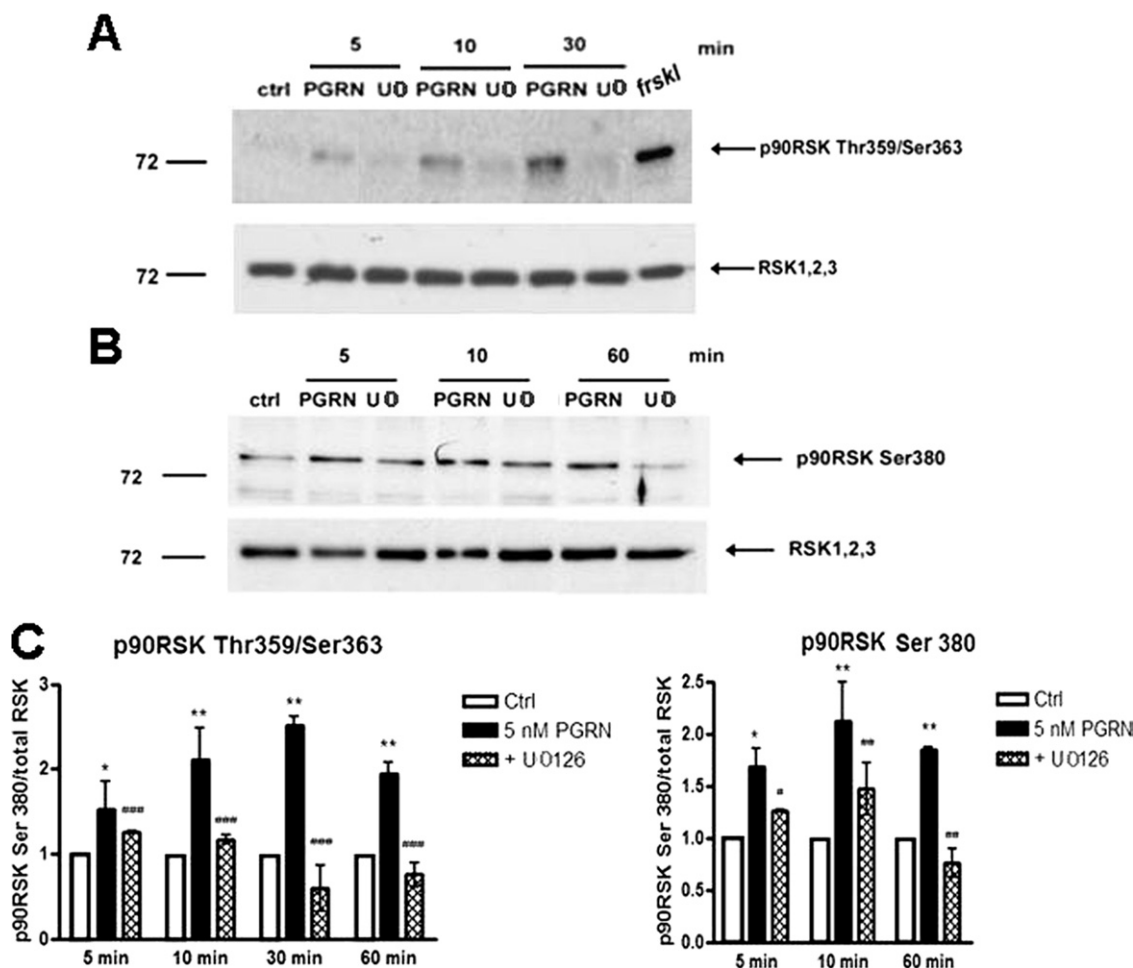
Fig. 4. PGRN stimulates phosphorylation of p90 ribosomal S6 kinase (p90RSK) in primary cortical neuronal cultures. (A) Rat neuronal cultures prepared as above were treated with 5 nM PGRN for the indicated time periods, in the absence or presence of MEK/ERK inhibitor U0126 (25 μM) and at the end of treatment, neurons were lysed and assayed by Western blotting for levels of p90RSK phosphorylated at Thr359/Ser363 and total RSK 1,2,3 proteins. (B) Cortical neurons were treated as in (A) for the indicated periods and levels of phosorylated p90RSK at Ser380 and total RSK 1,2,3 proteins were assayed as above. (C) Densitometric analysis of p90RSK Thr359/Ser363 and p90RSK Ser380 in neuronal cultures treated as above was performed and analyzed as described in Fig. 3. Data were obtained from 3 separate experiments (* $p < 0.05$, ** $p < 0.01$, one-way ANOVA followed by the Student-Newman-Keuls' test, comparing between cultures treated with PGRN and without treatment (ctrl); # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, comparing between cultures treated with PGRN in the presence and absence of U0126.

our neuronal cultures. Our data show that PGRN is remarkably stable in the media of neuronal cell cultures as we failed to detect any significant degradation of PGRN even after overnight incubation. Media PGRN was completely degraded by exogenous elastase suggesting neurons secrete little or no elastase or other PGRN processing enzymes to the medium (Fig. 2). Our observations support the hypothesis that the detected neuroprotective effects of exogenous PGRN are due to the full-length protein rather than its processing products granulins, although they do not exclude similar or parallel neuronal functions of these peptides. Our finding also suggests that secreted neuronal PGRN may act as an autocrine signaling factor to stimulate neuroprotection. Recent reports identify sortilin as a cell surface binding partner of PGRN indicating that this protein is internalized through sortilin-mediated endocytosis (Hu et al., 2010). It is

thus important to explore whether sortilin-mediated endocytosis of PGRN is involved in neuroprotection. Although our data show no decrease in the levels of extracellular PGRN, we cannot exclude the possibility that small and undetectable fractions of PGRN are endocytosed by neuronal cell surface sortilin. Alternatively, sortilin may be merely a regulator/controller of extracellular PGRN levels by endocytosis destined for lysosomal degradation, and the putative receptor for neuronal survival is yet to be found.

Exogenous PGRN rescued cell death in neuronal cultures subjected to toxic insults such as glutamate, H_2O_2 and MPP^+, but did not significantly alter the survival rate of cultures in the absence of toxic insults. Interestingly, the neuroprotective effects of PGRN, measured by different methodologies, were most pronounced against MPP^+-induced oxidative insult. MPP^+ is the active derivative of
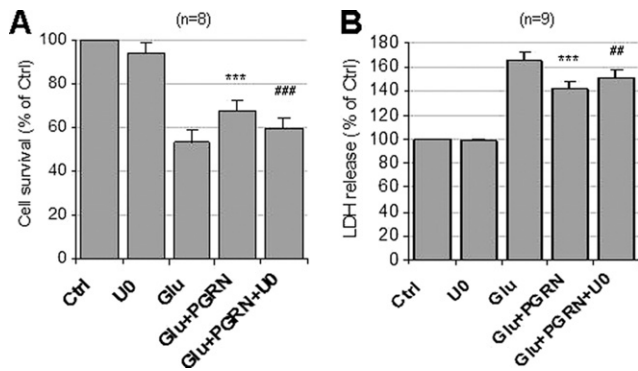
Fig. 5. Inhibition of ERK1/2 kinase blocks the neuroprotective function of extracellular PGRN against glutamate toxicity. (A) Rat cortical cultures of 8–12 DIV were pretreated for 1 hour with U0126 (10 μM) and then with 35 nM PGRN for 2 hours, followed by 50 μM glutamate for 24 hours. Cells were then fixed, stained and analyzed as described in the legend of Fig. 2A. Results (mean $\pm$ SE) were summarized from 8 independent experiments. *** $p < 0.001$ comparing cultures treated with glutamate in the presence and absence of PGRN (paired t test); ### $p < 0.001$ between cultures treated with PGRN/glutamate in the presence and absence of U0126 (paired t test). (B) Conditioned media of neuronal cultures prepared as above was replaced with fresh media and neurons were treated with U0126, PGRN, and glutamate as described above. Cell-free supernatants were then collected and LDH release was determined as described in the legend to Fig. 2C. Results (mean $\pm$ SE) were summarized from 9 independent experiments. *** $p < 0.001$ comparing between cultures treated with glutamate in the presence and absence of PGRN; ## $p < 0.01$ comparing between cultures treated with PGRN/glutamate in the presence and absence of U0126 (paired t test).

MPTP, a mitochondrial toxin that induces Parkinsonism in humans and experimental animals (Przedborski and Vila, 2003). The primary event in MPP+-induced cell death is the production of ROS with subsequent mitochondrial dysfunction and apoptotic death. On the other hand, glutamate-induced excitotoxicity is receptor-mediated and primarily involves Ca^{2+} overload followed by ROS generation and necrotic death. The differential effect of PGRN on neuronal survival of glutamate- or MPP+-treated cultures suggests that the neuroprotective effect of PGRN may depend on the specific cell death mechanism involved in each condition and that PGRN may be more potent against apoptotic than necrotic death. Furthermore, the potent protective effect of PGRN against the MPP+-induced neuronal cell death raises the possibility that PGRN reduces the release of reactive oxygen species through the inhibition of Complex I in the mitochondria and may affect neurodegenerative mechanisms specific to Parkinson's disease including degeneration of substantia nigra neurons. In this respect, it is of interest that PGRN has been proposed to be involved in Parkinson's disease (Brouwers et al., 2007; Sleegers et al., 2010).

Haploinsufficiency of PGRN in FTLD seems to necessitate a delicate mechanism to strictly maintain the extracellular levels of PGRN and recent reports suggest that PGRN-deficient hippocampal slices starved for glucose and oxygen show greater cell death compared with wild type tissues (Yin et al., 2010). Our data however, reveal specific neuronal signaling pathways regulated by PGRN. Furthermore, that increasing extracellular PGRN increases neuroprotection indicates that reduction of extracellular PGRN (like in FTLD) will decrease protection from toxic insults. In addition, our results reveal neuroprotective signaling stimulated by increased PGRN and this signaling should be less effective in conditions of reduced PGRN. Taken together, PGRN is not simply neurotrophic, but also neuroprotective against various noxious insults, a property that may be important to the survival of nonmitotic neuronal cells throughout life. Accordingly, compared with wild type neurons, neurons deficient in PGRN may be more vulnerable to chronic insults such as increased activity of the glutamatergic system or oxidative stress. Similar mechanism may apply to other neurodegenerative diseases as PGRN has been implicated in Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (Sleegers et al., 2010).

Treatment of neuronal cultures with PGRN increased phosphorylation of Akt and ERK kinases at epitopes associated with kinase activation and these phosphorylation events were sensitive to specific inhibitors of the MEK/ERK1/2 and PI3K/Akt signaling pathways (Fig. 3). Together, these data indicate that exogenous PGRN activates both the MEK/ERK1/2 and PI3K/Akt cell survival pathways of neuronal cells and suggest that these pathways may be involved in the PGRN-dependent neuroprotection against toxic insults. A recent report indicates that treatment of PGRN null (PGRN −/−) neurons with recombinant PGRN failed to stimulate phosphorylation of ERK1/2 (Kleinberger et al., 2010). Presently it is unclear whether this discrepancy is due to differences in the activities of the PGRN preparations used or to methodological differences in the protocols employed.

An important downstream effector of ERK1/2 is protein p90 ribosomal S6 kinase (p90RSK) which is phosphorylated and activated by ERK in the cytoplasm. Following activation, p90RSK translocates to the nucleus where it activates the serum response factor (SRF). Treatment of neuronal cultures with PGRN leads to the phosphorylation of specific p90RSK residues, a process inhibited by MEK/ERK inhibitor U0126 (Fig. 4). Because this inhibitor targets MEK, the upstream activator of ERK, our results indicate that exogenous PGRN stimulates the neuronal MEK/ERK/p90RSK cell signaling cascade and suggest that stimulation of this pathway contributes to the neuroprotective properties of PGRN. Indeed, inhibition of this cascade blocks the neuroprotective activity of PGRN against glutamate excitotoxicity (Fig. 5) further supporting the hypothesis that PGRN-induced activation of the MEK/ERK/p90RSK pathway plays important roles in the neuroprotective functions of this protein. Pharmacological inhibition of the MEK/ERK/p90RSK pathway also blocked the PGRN-dependent
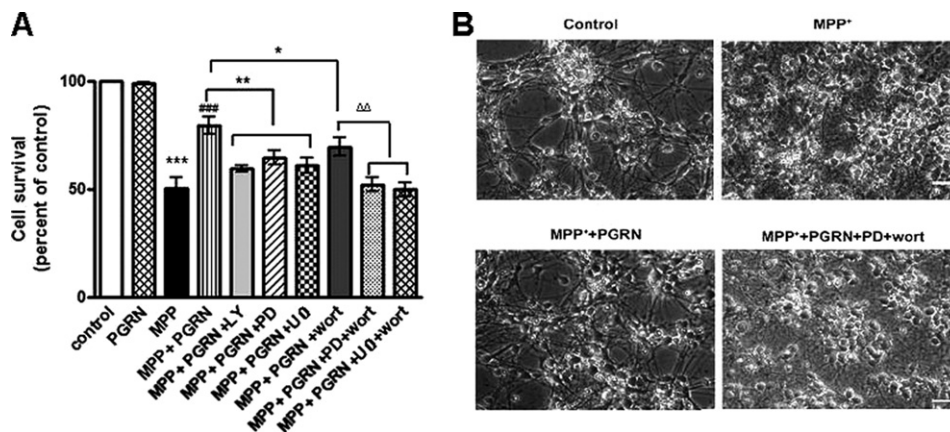
Fig. 6. ERK1/2 and Akt signaling mediate the neuroprotective activities of PGRN against MPP$^+$ toxicity. (A) Rat cortical cultures as in Fig. 1E were preincubated with PGRN (15 nM) for 30 minutes with or without inhibitors of MEK/ERK (U0126 or PD98059) or PI3K/Akt (wortmannin or LY-294002) signaling as indicated and then treated with 40 μM MPP$^+$ for 4 hours. Following MPP$^+$ treatment, cells were lysed with a nuclear sparing buffer. Intact nuclei were counted in a hemacytometer. Data are presented as mean $\pm$ SE, obtained from 3 separate experiments (one-way ANOVA followed by the Student-Newman-Keuls' test, *** $p < 0.001$, comparing between cultures treated with MPP$^+$ and untreated (control) or PGRN alone treated cultures; ### $p < 0.001$, comparing between MPP$^+$ and MPP$^+$+PGRN-treated cultures; * $p < 0.05$, ** $p < 0.01$, comparing between cultures treated with MPP$^+$+PGRN in the presence and absence of MEK/ERK1/2 and PI3K/Akt inhibitors; $^{\Delta\Delta}$ $p < 0.01$, comparing between cultures treated with MPP$^+$+PGRN+wortmannin in the presence and absence of PD98059 or U0126. (B) Representative photomicrographs of cortical neurons treated as in (A). MPP$^+$ treatment of neuronal cultures caused retraction of neuritic processes and eventually neuronal death (MPP$^+$). PGRN preserves the neuritic processes of MPP$^+$-treated cultures (MPP+PGRN) and this PGRN effect is abolished in the presence of MEK/ERK and PI3K/Akt inhibitors PD98059 and wortmannin, respectively. Magnification is 20×.

neuroprotection against neurotoxic agent MPP$^+$ (Fig. 6). In addition, inhibition of the PI3K/Akt signaling pathway using LY-294002 or wortmannin also blocked the PGRN-dependent neuroprotection against MPP$^+$. Interestingly, combined pharmacological inhibition of both pathways had a synergistic effect suppressing neuronal survival further than did inhibition of either pathway alone, suggesting that both pathways contribute to the neuroprotective activity of PGRN against MPP$^+$. Notably, the PGRN-conferred neuroprotection against MPP$^+$ was also characterized morphologically by sparing of neuronal processes (Fig. 6B). This PGRN effect is noteworthy especially in a postmitotic system of primary cortical neuronal cultures older than 7 DIV (Baki et al., 2008). Because MPP$^+$-induced death is characterized by axonal degeneration which proceeds soma demise (Przedborski and Vila, 2001), our results suggest that PGRN not only induces neurite outgrowth (Gao et al., 2010; Ryan et al., 2009; Van Damme et al., 2008) but also preserves integrity of neuronal processes (Fig. 6B). Together, our results support the hypothesis that reduction of functional PGRN in FTLD patients with PGRN mutations results in reduced activity of neuronal cell survival pathways such as MEK/ERK/p90RSK and PI3K/Akt thus decreasing neuronal protection against chronic toxic insults and leading to increased rates of neuronal cell death. That PGRN added to growth media acts as a neuroprotective factor against toxic insults suggests that cellular expression of PGRN may not be necessary for neuroprotection and that in vitro preparations of this protein may be used therapeutically if appropriately delivered to brain tissue, a hypothesis that needs experimental verification.

FTLD patients with PGRN mutations are often characterized by the presence of misfolded, polyubiquitinated, and abnormally phosphorylated C-terminal fragments of TAR DNA-binding protein 43 (TDP-43) in tau-negative and ubiquitin-positive neuronal inclusion bodies (Neumann et al., 2006; Sleegers et al., 2010). Recent reports suggest that neurons derived from PGRN-deficient mice accumulate phosphorylated TDP-43 fragments (Kleinberger et al., 2010; Yin et al., 2010) while suppressing PGRN induces a caspase-dependent cleavage of TDP-43, a process that may be relevant to accumulation of TDP-43 fragments (Kleinberger et al., 2010; Zhang et al., 2007). Thus, it would be important to explore whether the MEK/ERK/p90RSK and PI3K/Akt signaling pathways are involved in the molecular modifications that promote formation and translocation of TDP-43 aggregates. Deciphering the cellular signaling pathways that mediate the neuroprotective effects of PGRN and its involvement in the accumulation of abnormal aggregates common to the disease may lead to the development of new therapeutic interventions for the treatment of FTLD and related disorders.

Disclosure statement

The authors declare that there is no conflict of interest associated with this report.

All animal experiments were carried out in accordance with the rules and regulations at the Mount Sinai School of

Medicine and the Biomedical Research Foundation of the Academy of Athens.

Acknowledgements

We thank Dr. A. Bateman, McGill University, Canada, for human PGRN cDNA. This work was supported by NIH grants AG-17926, AG-08200, NS 047229 and Alzheimer's Association grant IIRG-10-174237.6.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2011.06.017.

References

Anjum, R., Blenis, J., 2008. The RSK family of kinases: emerging roles in cellular signalling. Nat. Rev. Mol. Cell Biol. 9, 747–758.

Arndt-Jovin, D.J., Jovin, T.M., 1977. Analysis and sorting of living cells according to deoxyribonucleic acid content. J. Histochem. Cytochem. 25, 585–589.

Baker, M., Mackenzie, I.R., Pickering-Brown, S.M., Gass, J., Rademakers, R., Lindholm, C., Snowden, J., Adamson, J., Sadovnick, A.D., Rollinson, S., Cannon, A., Dwosh, E., Neary, D., Melquist, S., Richardson, A., Dickson, D., Berger, Z., Eriksen, J., Robinson, T., Zehr, C., Dickey, C.A., Crook, R., McGowan, E., Mann, D., Boeve, B., Feldman, H., Hutton, M., 2006. Mutations in progranulin cause tau-negative frontotemporal dementia linked to chromosome 17. Nature 442, 916–919.

Baki, L., Neve, R.L., Shao, Z., Shioi, J., Georgakopoulos, A., Robakis, N.K., 2008. Wild-type but not FAD mutant presenilin-1 prevents neuronal degeneration by promoting phosphatidylinositol 3-kinase neuroprotective signaling. J. Neurosci. 28, 483–490.

Bateman, A., Bennett, H.P., 2009. The granulin gene family: from cancer to dementia. Bioessays 31, 1245–1254.

Brouwers, N., Nuytemans, K., van der Zee, J., Gijselinck, I., Engelborghs, S., Theuns, J., Kumar-Singh, S., Pickut, B.A., Pals, P., Dermaut, B., Bogaerts, V., De Pooter, T., Serneels, S., Van den Broeck, M., Cuijt, I., Mattheijssens, M., Peeters, K., Sciot, R., Martin, J.J., Cras, P., Santens, P., Vandenberghe, R., De Deyn, P.P., Cruts, M., Van Broeckhoven, C., Sleegers, K., 2007. Alzheimer and Parkinson diagnoses in progranulin null mutation carriers in an extended founder family. Arch. Neurol. 64, 1436–1446.

Butler, G.S., Dean, R.A., Tam, E.M., Overall, C.M., 2008. Pharmacoproteomics of a metalloproteinase hydroxamate inhibitor in breast cancer cells: dynamics of membrane type 1 matrix metalloproteinase-mediated membrane protein shedding. Mol. Cell. Biol. 28, 4896–4914.

Cruts, M., Gijselinck, I., van der Zee, J., Engelborghs, S., Wils, H., Pirici, D., Rademakers, R., Vandenberghe, R., Dermaut, B., Martin, J.J., van Duijn, C., Peeters, K., Sciot, R., Santens, P., De Pooter, T., Mattheijssens, M., Van den Broeck, M., Cuijt, I., Vennekens, K., De Deyn, P.P., Kumar-Singh, S., Van Broeckhoven, C., 2006. Null mutations in progranulin cause ubiquitin-positive frontotemporal dementia linked to chromosome 17q21. Nature 442, 920–924.

Daniel, R., He, Z., Carmichael, K.P., Halper, J., Bateman, A., 2000. Cellular localization of gene expression for progranulin. J. Histochem. Cytochem. 48, 999–1009.

Denizot, F., Lang, R., 1986. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods 89, 271–277.

Farinelli, S.E., Greene, L.A., Friedman, W.J., 1998. Neuroprotective actions of dipyridamole on cultured CNS neurons. J. Neurosci. 18, 5112–5123.

Fatokun, A.A., Stone, T.W., Smith, R.A., 2008. Oxidative stress in neurodegeneration and available means of protection. Front. Biosci. 13, 3288–3311.

Fayard, E., Tintignac, L.A., Baudry, A., Hemmings, B.A., 2005. Protein kinase B/Akt at a glance. J. Cell Sci. 118, 5675–5678.

Finch, N., Baker, M., Crook, R., Swanson, K., Kuntz, K., Surtees, R., Bisceglio, G., Rovelet-Lecrux, A., Boeve, B., Petersen, R.C., Dickson, D.W., Younkin, S.G., Deramecourt, V., Crook, J., Graff-Radford, N.R., Rademakers, R., 2009. Plasma progranulin levels predict progranulin mutation status in frontotemporal dementia patients and asymptomatic family members. Brain 132, 583–591.

Gao, X., Joselin, A.P., Wang, L., Kar, A., Ray, P., Bateman, A., Goate, A.M., Wu, J.Y., 2010. Progranulin promotes neurite outgrowth and neuronal differentiation by regulating GSK-3beta. Protein Cell. 1, 552–562.

Ghidoni, R., Benussi, L., Glionna, M., Franzoni, M., Binetti, G., 2008. Low plasma progranulin levels predict progranulin mutations in frontotemporal lobar degeneration. Neurology 71, 1235–1239.

Gijselinck, I., Van Broeckhoven, C., Cruts, M., 2008. Granulin mutations associated with frontotemporal lobar degeneration and related disorders: An update. Hum. Mutat. 29, 1373–1386.

He, Z., Bateman, A., 2003. Progranulin (granulin-epithelin precursor, PC-cell-derived growth factor, acrogranin) mediates tissue repair and tumorigenesis. J. Mol. Med. 81, 600–612.

He, Z., Ismail, A., Kriazhev, L., Sadvakassova, G., Bateman, A., 2002. Progranulin (PC-cell-derived growth factor/acrogranin) regulates invasion and cell survival. Cancer Res. 62, 5590–5596.

Hu, F., Padukkavidana, T., Vægter, C.B., Brady, O.A., Zheng, Y., Mackenzie, I.R., Feldman, H.H., Nykjaer, A., Strittmatter, S.M., 2010. Sortilin-mediated endocytosis determines levels of the frontotemporal dementia protein, progranulin. Neuron 68, 654–667.

Kessenbrock, K., Fröhlich, L., Sixt, M., Lämmermann, T., Pfister, H., Bateman, A., Belaaouaj, A., Ring, J., Ollert, M., Fässler, R., Jenne, D.E., 2008. Proteinase 3 and neutrophil elastase enhance inflammation in mice by inactivating antiinflammatory progranulin. J. Clin. Invest. 118, 2438–2447.

Kleinberger, G., Wils, H., Ponsaerts, P., Joris, G., Timmermans, J.P., Van Broeckhoven, C., Kumar-Singh, S., 2010. Increased caspase activation and decreased TDP-43 solubility in progranulin knockout cortical cultures. J. Neurochem. 115, 735–747.

Koh, J.Y., Choi, D.W., 1987. Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. J. Neurosci. Methods 20, 83–90.

Laird, A.S., Van Hoecke, A., De Muynck, L., Timmers, M., Van den Bosch, L., Van Damme, P., Robberecht, W., 2010. Progranulin is neurotrophic in vivo and protects against a mutant TDP-43 induced axonopathy. PLoS One 5, e13368.

Lau, A., Tymianski, M., 2010. Glutamate receptors, neurotoxicity and neurodegeneration. Pflugers Arch. 460, 525–542.

Lu, R., Serrero, G., 2001. Mediation of estrogen mitogenic effect in human breast cancer MCF-7 cells by PC-cell-derived growth factor (PCDGF/granulin precursor). Proc. Natl. Acad. Sci. U. S. A. 98, 142–147.

Matsuwaki, T., Asakura, R., Suzuki, M., Yamanouchi, K., Nishihara, M., 2011. Age-dependent changes in progranulin expression in the mouse brain. J. Reprod. Dev. 57, 113–119.

Monami, G., Gonzalez, E.M., Hellman, M., Gomella, L.G., Baffa, R., Iozzo, R.V., Morrione, A., 2006. Proepithelin promotes migration and invasion of 5637 bladder cancer cells through the activation of ERK1/2 and the formation of a paxillin/FAK/ERK complex. Cancer Res. 66, 7103–7110.

Neumann, M., Sampathu, D.M., Kwong, L.K., Truax, A.C., Micsenyi, M.C., Chou, T.T., Bruce, J., Schuck, T., Grossman, M., Clark, C.M., McCluskey, L.F., Miller, B.L., Masliah, E., Mackenzie, I.R., Feldman, H., Feiden, W., Kretzschmar, H.A., Trojanowski, J.Q., Lee, V.M., 2006. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Science 314, 130–133.

2326.e16

Paxinou, E., Chen, Q., Weisse, M., Giasson, B.I., Norris, E.H., Rueter, S.M., Trojanowski, J.Q., Lee, V.M., Ischiropoulos, H., 2001. Induction of alpha-synuclein aggregation by intracellular nitrative insult. J. Neurosci. 21, 8053–8061.

Payne, D.M., Rossomando, A.J., Martino, P., Erickson, A.K., Her, J.H., Shabanowitz, J., Hunt, D.F., Weber, M.J., Sturgill, T.W., 1991. Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). EMBO J. 10, 885–892.

Plowman, G.D., Green, J.M., Neubauer, M.G., Buckley, S.D., McDonald, V.L., Todaro, G.J., Shoyab, M., 1992. The epithelin precursor encodes two proteins with opposing activities on epithelial cell growth. J. Biol. Chem. 267, 13073–13078.

Przedborski, S., Vila, M., 2001. The last decade in Parkinson's disease research. Basic sciences. Adv. Neurol. 86, 177–186.

Przedborski, S., Vila, M., 2003. The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model: a tool to explore the pathogenesis of Parkinson's disease. Ann. N. Y. Acad. Sci. 991, 189–198.

Ryan, C.L., Baranowski, D.C., Chitramuthu, B.P., Malik, S., Li, Z., Cao, M., Minotti, S., Durham, H.D., Kay, D.G., Shaw, C.A., Bennett, H.P., Bateman, A., 2009. Progranulin is expressed within motor neurons and promotes neuronal cell survival. BMC Neurosci. 10, 130.

Shankaran, S.S., Capell, A., Hruscha, A.T., Fellerer, K., Neumann, M., Schmid, B., Haass, C., 2008. Missense mutations in the progranulin gene linked to frontotemporal lobar degeneration with ubiquitin-immunoreactive inclusions reduce progranulin production and secretion. J. Biol. Chem. 283, 1744–1753.

Sleegers, K., Brouwers, N., Van Damme, P., Engelborghs, S., Gijselinck, I., van der Zee, J., Peeters, K., Mattheijssens, M., Cruts, M., Vandenberghe, R., De Deyn, P.P., Robberecht, W., Van Broeckhoven, C., 2009. Serum biomarker for progranulin-associated frontotemporal lobar degeneration. Ann. Neurol. 65, 603–609.

Sleegers, K., Cruts, M., Van Broeckhoven, C., 2010. Molecular pathways of frontotemporal lobar degeneration. Annu. Rev. Neurosci. 33, 71–88.

Stefanis, L., Park, D.S., Friedman, W.J., Greene, L.A., 1999. Caspase-dependent and -independent death of camptothecin-treated embryonic cortical neurons. J. Neurosci. 19, 6235–6247.

Tolkatchev, D., Malik, S., Vinogradova, A., Wang, P., Chen, Z., Xu, P., Bennett, H.P., Bateman, A., Ni, F., 2008. Structure dissection of human progranulin identifies well-folded granulin/epithelin modules with unique functional activities. Protein Sci. 17, 711–724.

Van Damme, P., Van Hoecke, A., Lambrechts, D., Vanacker, P., Bogaert, E., van Swieten, J., Carmeliet, P., Van Den Bosch, L., Robberecht, W., 2008. Progranulin functions as a neurotrophic factor to regulate neurite outgrowth and enhance neuronal survival. J. Cell Biol. 181, 37–41.

Vogiatzi, T., Xilouri, M., Vekrellis, K., Stefanis, L., 2008. Wild type alpha-synuclein is degraded by chaperone-mediated autophagy and macroautophagy in neuronal cells. J. Biol. Chem. 283, 23542–23556.

Wang, J., Van Damme, P., Cruchaga, C., Gitcho, M.A., Vidal, J.M., Seijo-Martínez, M., Wang, L., Wu, J.Y., Robberecht, W., Goate, A., 2010. Pathogenic cysteine mutations affect progranulin function and production of mature granulins. J. Neurochem. 112, 1305–1315.

Xu, J., Litterst, C., Georgakopoulos, A., Zaganas, I., Robakis, N.K., 2009. Peptide EphB2/CTF2 generated by the gamma-secretase processing of EphB2 receptor promotes tyrosine phosphorylation and cell surface localization of N-methyl-D-aspartate receptors. J. Biol. Chem. 284, 27220–27228.

Yin, F., Banerjee, R., Thomas, B., Zhou, P., Qian, L., Jia, T., Ma, X., Ma, Y., Iadecola, C., Beal, M.F., Nathan, C., Ding, A., 2010. Exaggerated inflammation, impaired host defense, and neuropathology in progranulin-deficient mice. J. Exp. Med. 207, 117–128.

Zanocco-Marani, T., Bateman, A., Romano, G., Valentinis, B., He, Z.H., Baserga, R., 1999. Biological activities and signaling pathways of the granulin/epithelin precursor. Cancer Res. 59, 5331–5340.

Zhang, Y.J., Xu, Y.F., Dickey, C.A., Buratti, E., Baralle, F., Bailey, R., Pickering-Brown, S., Dickson, D., Petrucelli, L., 2007. Progranulin mediates caspase-dependent cleavage of TAR DNA binding protein-43. J. Neurosci. 27, 10530–10534.

Zhu, J., Nathan, C., Jin, W., Sim, D., Ashcroft, G.S., Wahl, S.M., Lacomis, L., Erdjument-Bromage, H., Tempst, P., Wright, C.D., Ding, A., 2002. Conversion of proepithelin to epithelins: roles of SLPI and elastase in host defense and wound repair. Cell 111, 867–878.
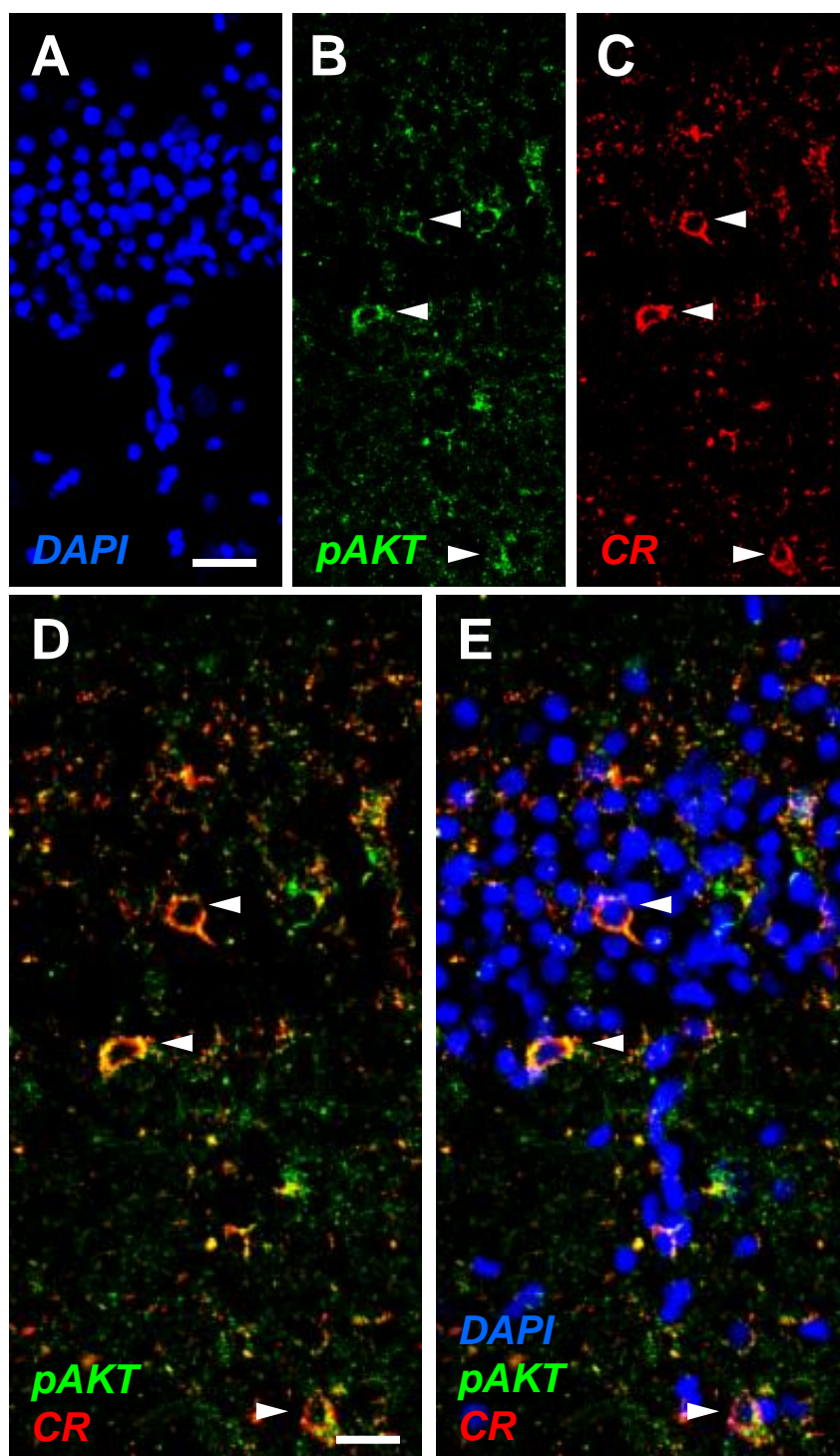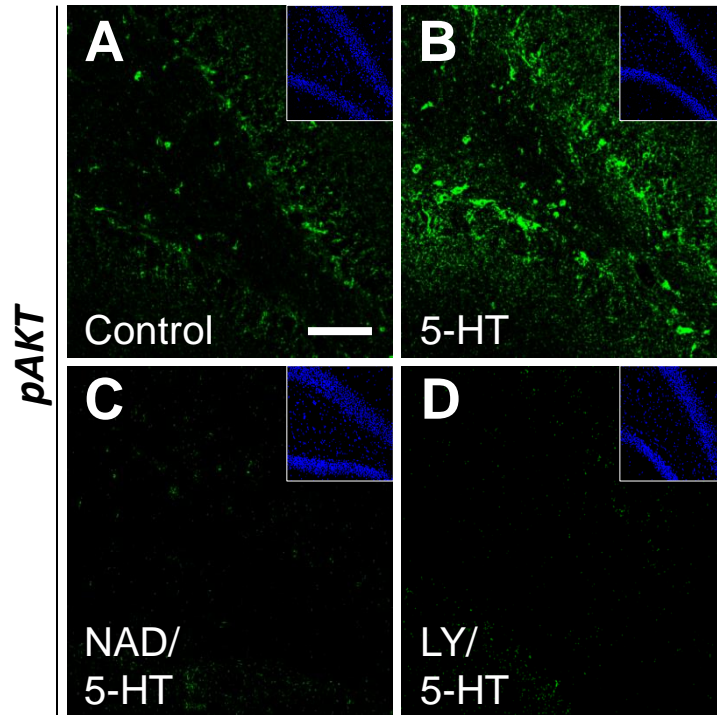
Immunohistochemical detection of **pAkt** (green) and **calretinin** (red), a calcium binding protein, in the dentate gyrus of rats fed a chow diet after stimulation of brain slices with 5-HT (10 µM). Arrowheads indicate the colocalizations (orange/yellow). Counterstaining with DAPI (blue).
Confocal laser scanning. Scale bar: 30 µm.
CR, calretinin.

Annex 3

Immunohistochemical detection of **pAkt** (green) in the dentate gyrus of rats fed a chow diet after stimulation of brain slices without **(A)** or with **(B-D)** 5-HT (10 µM). Some 5HT-stimulated slices were pre-treated for 20 min and then co-treated with either **(C)** NAD 299 hydrochloride (10 mM, Tocris bioscience, Bristol, UK), a 5-HT$_{1A}$ antagonist, or **(D)** LY294002 (10 µM, Sigma), a PI3K inhibitor. Theses pre-treatments prevented 5-HT from inducing the phosphorylation of Akt. Control slices were incubated with buffer alone **(A)**. Corresponding DAPI counterstained sections (blue) are shown on the right top corner of each picture. Confocal laser scanning. Scale bar: 135 µm.

Annex 4
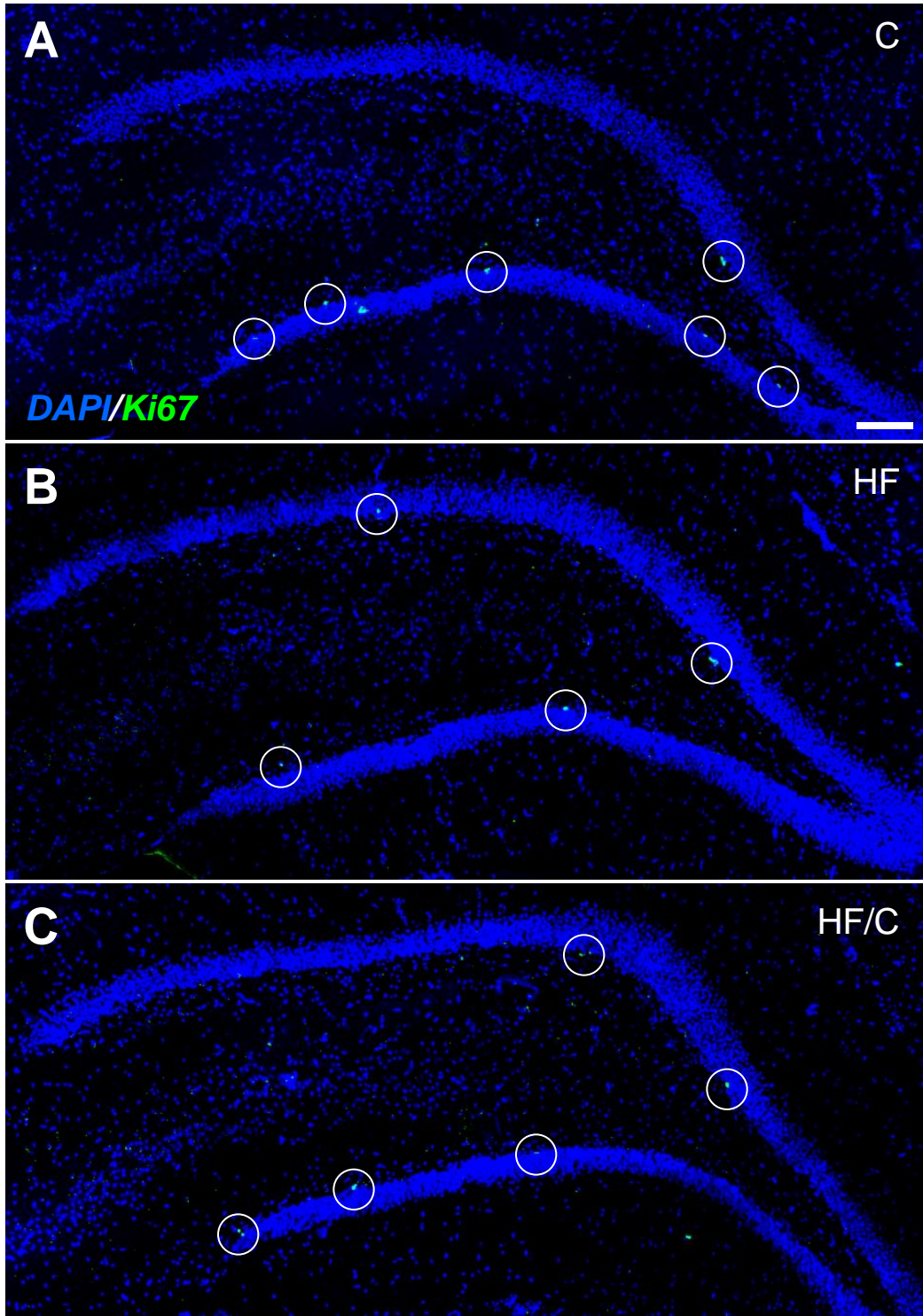
Immunohistochemical detection of **Ki-67** (green), a nuclear cell proliferation marker, in the dentate gyrus of rats fed **(A)** a chow diet (C), **(B)** a HF diet (HF) or **(C)** HF reversed to chow diet (HF/C). Circles indicate the localizations of Ki-67-positive cells on DAPI (blue) counterstained sections. The HF diet decreased the number of Ki-67-positive cells in the dentate gyrus of rats, which was partially reversed with chow diet restoration.
Confocal laser scanning. Scale bar: 150 μm.
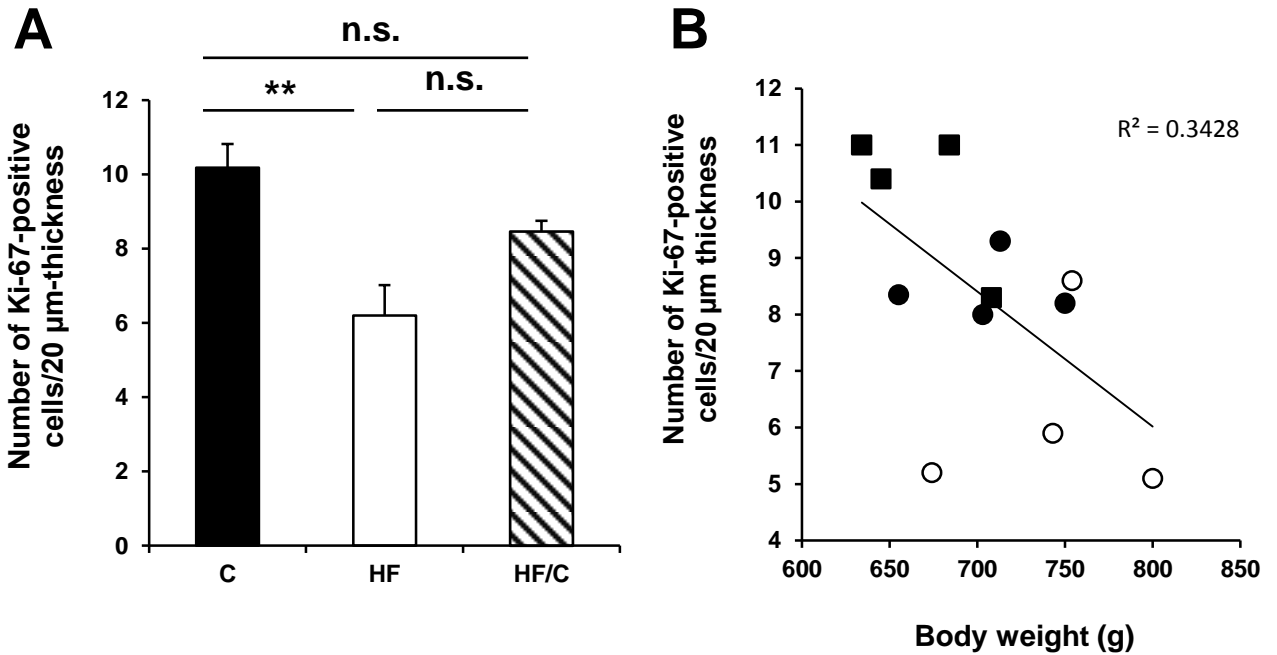
(A) Quantification of the immunohistochemical detection of **Ki-67** in the dentate gyrus of rats fed a chow diet (C), a HF diet (HF) or HF reversed to chow diet (HF/C). Data are presented as average number of cells over 20-µm-thick dentate gyrus. The HF diet decreased the number of Ki-67-positive cells by ~40% in the dentate gyrus of rats, which was partially reversed with chow diet restoration. n.s., non significant; **p<0.01. **(B)** Inverse correlation between the average number of Ki-67-positive cells and the body weight of rats fed a chow diet (closed boxes), a HF diet (open circles) or HF reversed to chow diet (closed circles). p<0.05.

REFERENCES

REFERENCES

Al-Khouri, A.M., Y. Ma, S.H. Togo, S. Williams, and T. Mustelin. 2005. Cooperative phosphorylation of the tumor suppressor phosphatase and tensin homologue (PTEN) by casein kinases and glycogen synthase kinase 3beta. J Biol Chem. 280:35195-35202.

Al-Qassab, H., M.A. Smith, E.E. Irvine, J. Guillermet-Guibert, M. Claret, A.I. Choudhury, C. Selman, K. Piipari, M. Clements, S. Lingard, K. Chandarana, J.D. Bell, G.S. Barsh, A.J. Smith, R.L. Batterham, M.L. Ashford, B. Vanhaesebroeck, and D.J. Withers. 2009. Dominant role of the p110beta isoform of PI3K over p110alpha in energy homeostasis regulation by POMC and AgRP neurons. Cell Metab. 10:343-354.

Albu, J.B., L.K. Heilbronn, D.E. Kelley, S.R. Smith, K. Azuma, E.S. Berk, F.X. Pi-Sunyer, and E. Ravussin. 2010. Metabolic changes following a 1-year diet and exercise intervention in patients with type 2 diabetes. Diabetes. 59:627-633.

Alessi, D.R., M. Andjelkovic, B. Caudwell, P. Cron, N. Morrice, P. Cohen, and B.A. Hemmings. 1996. Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J. 15:6541-6551.

Alessi, D.R., S.R. James, C.P. Downes, A.B. Holmes, P.R. Gaffney, C.B. Reese, and P. Cohen. 1997. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol. 7:261-269.

Anhe, G.F., L.C. Caperuto, M. Pereira-Da-Silva, L.C. Souza, A.E. Hirata, L.A. Velloso, J. Cipolla-Neto, and C.R. Carvalho. 2004. In vivo activation of insulin receptor tyrosine kinase by melatonin in the rat hypothalamus. J Neurochem. 90:559-566.

Azmitia, E.C., and M. Segal. 1978. An autoradiographic analysis of the differential ascending projections of the dorsal and median raphe nuclei in the rat. J Comp Neurol. 179:641-667.

Backer, J.M. 2008. The regulation and function of Class III PI3Ks: novel roles for Vps34. Biochem J. 410:1-17.

Baganz, N.L., R.E. Horton, A.S. Calderon, W.A. Owens, J.L. Munn, L.T. Watts, N. Koldzic-Zivanovic, N.A. Jeske, W. Koek, G.M. Toney, and L.C. Daws. 2008. Organic cation transporter 3: Keeping the brake on extracellular serotonin in serotonin-transporter-deficient mice. Proc Natl Acad Sci U S A. 105:18976-18981.

Balthasar, N., R. Coppari, J. McMinn, S.M. Liu, C.E. Lee, V. Tang, C.D. Kenny, R.A. McGovern, S.C. Chua, Jr., J.K. Elmquist, and B.B. Lowell. 2004. Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis. Neuron. 42:983-991.

Balthasar, N., L.T. Dalgaard, C.E. Lee, J. Yu, H. Funahashi, T. Williams, M. Ferreira, V. Tang, R.A. McGovern, C.D. Kenny, L.M. Christiansen, E. Edelstein, B. Choi, O. Boss, C. Aschkenasi, C.Y. Zhang, K. Mountjoy, T. Kishi, J.K. Elmquist, and B.B. Lowell. 2005. Divergence of melanocortin pathways in the control of food intake and energy expenditure. Cell. 123:493-505.

Banks, W.A., and A.J. Kastin. 1998. Differential permeability of the blood-brain barrier to two pancreatic peptides: insulin and amylin. Peptides. 19:883-889.

Banks, W.A., A.J. Kastin, W. Huang, J.B. Jaspan, and L.M. Maness. 1996. Leptin enters the brain by a saturable system independent of insulin. Peptides. 17:305-311.

Banks, W.A., J.B. Owen, and M.A. Erickson. 2012. Insulin in the brain: there and back again. Pharmacol Ther. 136:82-93.

Banting, F.G., C.H. Best, J.B. Collip, W.R. Campbell, and A.A. Fletcher. 1922. Pancreatic Extracts in the Treatment of Diabetes Mellitus. Can Med Assoc J. 12:141-146.

Bardin, L. 2011. The complex role of serotonin and 5-HT receptors in chronic pain. Behav Pharmacol. 22:390-404.

Barone, P., D. Jordan, F. Atger, N. Kopp, and G. Fillion. 1994. Quantitative autoradiography of 5-HT1D and 5-HT1E binding sites labelled by [3H]5-HT, in frontal cortex and the hippocampal region of the human brain. Brain Res. 638:85-94.

Bartholomeusz, C., and A.M. Gonzalez-Angulo. 2012. Targeting the PI3K signaling pathway in cancer therapy. Expert Opin Ther Targets. 16:121-130.

Bass, J., G. Chiu, Y. Argon, and D.F. Steiner. 1998. Folding of insulin receptor monomers is facilitated by the molecular chaperones calnexin and calreticulin and impaired by rapid dimerization. J Cell Biol. 141:637-646.

Beaudet, A., and L. Descarries. 1979. Radioautographic characterization of a serotonin-accumulating nerve cell group in adult rat hypothalamus. Brain Res. 160:231-243.

Beaulieu, J.M., T.D. Sotnikova, S. Marion, R.J. Lefkowitz, R.R. Gainetdinov, and M.G. Caron. 2005. An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. Cell. 122:261-273.

Beck, S.G., K.C. Choi, and T.J. List. 1992. Comparison of 5-hydroxytryptamine1A-mediated hyperpolarization in CA1 and CA3 hippocampal pyramidal cells. J Pharmacol Exp Ther. 263:350-359.

Begg, D.P., J.D. Mul, M. Liu, B.M. Reedy, D.A. D'Alessio, R.J. Seeley, and S.C. Woods. 2013. Reversal of diet-induced obesity increases insulin transport into cerebrospinal fluid and restores sensitivity to the anorexic action of central insulin in male rats. Endocrinology. 154:1047-1054.

Belfiore, A., F. Frasca, G. Pandini, L. Sciacca, and R. Vigneri. 2009. Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. Endocr Rev. 30:586-623.

Belgardt, B.F., and J.C. Bruning. 2010. CNS leptin and insulin action in the control of energy homeostasis. Ann N Y Acad Sci. 1212:97-113.

Belgardt, B.F., A. Husch, E. Rother, M.B. Ernst, F.T. Wunderlich, B. Hampel, T. Klockener, D. Alessi, P. Kloppenburg, and J.C. Bruning. 2008. PDK1 deficiency in POMC-expressing cells reveals FOXO1-

dependent and -independent pathways in control of energy homeostasis and stress response. Cell Metab. 7:291-301.

Bell, G.I., R.L. Pictet, W.J. Rutter, B. Cordell, E. Tischer, and H.M. Goodman. 1980. Sequence of the human insulin gene. Nature. 284:26-32.

Belmaker, R.H., and G. Agam. 2008. Major depressive disorder. N Engl J Med. 358:55-68.

Benomar, Y., F. Berthou, C.M. Vacher, V. Bailleux, A. Gertler, J. Djiane, and M. Taouis. 2009. Leptin but not ciliary neurotrophic factor (CNTF) induces phosphotyrosine phosphatase-1B expression in human neuronal cells (SH-SY5Y): putative explanation of CNTF efficacy in leptin-resistant state. Endocrinology. 150:1182-1191.

Benomar, Y., A.F. Roy, A. Aubourg, J. Djiane, and M. Taouis. 2005a. Cross down-regulation of leptin and insulin receptor expression and signalling in a human neuronal cell line. Biochem J. 388:929-939.

Benomar, Y., S. Wetzler, C. Larue-Achagiotis, J. Djiane, D. Tome, and M. Taouis. 2005b. In vivo leptin infusion impairs insulin and leptin signalling in liver and hypothalamus. Mol Cell Endocrinol. 242:59-66.

Benyoucef, S., K.H. Surinya, D. Hadaschik, and K. Siddle. 2007. Characterization of insulin/IGF hybrid receptors: contributions of the insulin receptor L2 and Fn1 domains and the alternatively spliced exon 11 sequence to ligand binding and receptor activation. Biochem J. 403:603-613.

Benzler, J., G.K. Ganjam, M. Kruger, O. Pinkenburg, M. Kutschke, S. Stohr, J. Steger, C.E. Koch, R. Olkrug, M.W. Schwartz, P.R. Shepherd, D.R. Grattan, and A. Tups. 2012. Hypothalamic glycogen synthase kinase 3beta has a central role in the regulation of food intake and glucose metabolism. Biochem J. 447:175-184.

Berton, O., and E.J. Nestler. 2006. New approaches to antidepressant drug discovery: beyond monoamines. Nat Rev Neurosci. 7:137-151.

Berumen, L.C., A. Rodriguez, R. Miledi, and G. Garcia-Alcocer. 2012. Serotonin receptors in hippocampus. ScientificWorldJournal. 2012:823493.

Bhatnagar, S., N. Nowak, L. Babich, and L. Bok. 2004. Deletion of the 5-HT3 receptor differentially affects behavior of males and females in the Porsolt forced swim and defensive withdrawal tests. Behav Brain Res. 153:527-535.

Bielas, S.L., J.L. Silhavy, F. Brancati, M.V. Kisseleva, L. Al-Gazali, L. Sztriha, R.A. Bayoumi, M.S. Zaki, A. Abdel-Aleem, R.O. Rosti, H. Kayserili, D. Swistun, L.C. Scott, E. Bertini, E. Boltshauser, E. Fazzi, L. Travaglini, S.J. Field, S. Gayral, M. Jacoby, S. Schurmans, B. Dallapiccola, P.W. Majerus, E.M. Valente, and J.G. Gleeson. 2009. Mutations in INPP5E, encoding inositol polyphosphate-5-phosphatase E, link phosphatidyl inositol signaling to the ciliopathies. Nat Genet. 41:1032-1036.

Biggs, W.H., 3rd, J. Meisenhelder, T. Hunter, W.K. Cavenee, and K.C. Arden. 1999. Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. Proc Natl Acad Sci U S A. 96:7421-7426.

Bjorbaek, C., K. El-Haschimi, J.D. Frantz, and J.S. Flier. 1999. The role of SOCS-3 in leptin signaling and leptin resistance. J Biol Chem. 274:30059-30065.

Bjorbaek, C., J.K. Elmquist, P. Michl, R.S. Ahima, A. van Bueren, A.L. McCall, and J.S. Flier. 1998. Expression of leptin receptor isoforms in rat brain microvessels. Endocrinology. 139:3485-3491.

Bjorbak, C., H.J. Lavery, S.H. Bates, R.K. Olson, S.M. Davis, J.S. Flier, and M.G. Myers, Jr. 2000. SOCS3 mediates feedback inhibition of the leptin receptor via Tyr985. J Biol Chem. 275:40649-40657.

Bjornholm, M., H. Munzberg, R.L. Leshan, E.C. Villanueva, S.H. Bates, G.W. Louis, J.C. Jones, R. Ishida-Takahashi, C. Bjorbaek, and M.G. Myers, Jr. 2007. Mice lacking inhibitory leptin receptor signals are lean with normal endocrine function. J Clin Invest. 117:1354-1360.

Blackburn, K.J., P.C. French, and R.J. Merrills. 1967. 5-hydroxytryptamine uptake by rat brain in vitro. Life Sci. 6:1653-1663.

Blakely, R.D., H.E. Berson, R.T. Fremeau, Jr., M.G. Caron, M.M. Peek, H.K. Prince, and C.C. Bradley. 1991. Cloning and expression of a functional serotonin transporter from rat brain. Nature. 354:66-70.

Blondeau, F., J. Laporte, S. Bodin, G. Superti-Furga, B. Payrastre, and J.L. Mandel. 2000. Myotubularin, a phosphatase deficient in myotubular myopathy, acts on phosphatidylinositol 3-kinase and phosphatidylinositol 3-phosphate pathway. Hum Mol Genet. 9:2223-2229.

Bockaert, J., S. Claeysen, C. Becamel, A. Dumuis, and P. Marin. 2006. Neuronal 5-HT metabotropic receptors: fine-tuning of their structure, signaling, and roles in synaptic modulation. Cell Tissue Res. 326:553-572.

Bogers, R.P., W.J. Bemelmans, R.T. Hoogenveen, H.C. Boshuizen, M. Woodward, P. Knekt, R.M. van Dam, F.B. Hu, T.L. Visscher, A. Menotti, R.J. Thorpe, Jr., K. Jamrozik, S. Calling, B.H. Strand, and M.J. Shipley. 2007. Association of overweight with increased risk of coronary heart disease partly independent of blood pressure and cholesterol levels: a meta-analysis of 21 cohort studies including more than 300 000 persons. Arch Intern Med. 167:1720-1728.

Bondy, C.A., and C.M. Cheng. 2004. Signaling by insulin-like growth factor 1 in brain. Eur J Pharmacol. 490:25-31.

Bortolozzi, A., A. Castane, J. Semakova, N. Santana, G. Alvarado, R. Cortes, A. Ferres-Coy, G. Fernandez, M.C. Carmona, M. Toth, J.C. Perales, A. Montefeltro, and F. Artigas. 2012. Selective siRNA-mediated suppression of 5-HT1A autoreceptors evokes strong anti-depressant-like effects. Mol Psychiatry. 17:612-623.

Braccini, L., E. Ciraolo, M. Martini, T. Pirali, G. Germena, K. Rolfo, and E. Hirsch. 2012. PI3K keeps the balance between metabolism and cancer. Adv Biol Regul. 52:389-405.

Bray, G.A. 2002. The underlying basis for obesity: relationship to cancer. J Nutr. 132:3451S-3455S.

Breisch, S.T., F.P. Zemlan, and B.G. Hoebel. 1976. Hyperphagia and obesity following serotonin depletion by intraventricular p-chlorophenylalanine. Science. 192:382-385.

Brock, C., M. Schaefer, H.P. Reusch, C. Czupalla, M. Michalke, K. Spicher, G. Schultz, and B. Nurnberg. 2003. Roles of G beta gamma in membrane recruitment and activation of p110 gamma/p101 phosphoinositide 3-kinase gamma. J Cell Biol. 160:89-99.

Brodbeck, D., P. Cron, and B.A. Hemmings. 1999. A human protein kinase Bgamma with regulatory phosphorylation sites in the activation loop and in the C-terminal hydrophobic domain. J Biol Chem. 274:9133-9136.

Buescher, J.L., and C.J. Phiel. 2010. A noncatalytic domain of glycogen synthase kinase-3 (GSK-3) is essential for activity. J Biol Chem. 285:7957-7963.

Buhot, M.C. 1997. Serotonin receptors in cognitive behaviors. Curr Opin Neurobiol. 7:243-254.

Buhot, M.C., S. Martin, and L. Segu. 2000. Role of serotonin in memory impairment. Ann Med. 32:210-221.

Burke, J.E., O. Vadas, A. Berndt, T. Finegan, O. Perisic, and R.L. Williams. 2011. Dynamics of the phosphoinositide 3-kinase p110delta interaction with p85alpha and membranes reveals aspects of regulation distinct from p110alpha. Structure. 19:1127-1137.

Burns, C.M., H. Chu, S.M. Rueter, L.K. Hutchinson, H. Canton, E. Sanders-Bush, and R.B. Emeson. 1997. Regulation of serotonin-2C receptor G-protein coupling by RNA editing. Nature. 387:303-308.

Cabrele, C., M. Langer, R. Bader, H.A. Wieland, H.N. Doods, O. Zerbe, and A.G. Beck-Sickinger. 2000. The first selective agonist for the neuropeptide YY5 receptor increases food intake in rats. J Biol Chem. 275:36043-36048.

Calleja, V., D. Alcor, M. Laguerre, J. Park, B. Vojnovic, B.A. Hemmings, J. Downward, P.J. Parker, and B. Larijani. 2007. Intramolecular and intermolecular interactions of protein kinase B define its activation in vivo. PLoS Biol. 5:e95.

Canton, H., R.B. Emeson, E.L. Barker, J.R. Backstrom, J.T. Lu, M.S. Chang, and E. Sanders-Bush. 1996. Identification, molecular cloning, and distribution of a short variant of the 5-hydroxytryptamine2C receptor produced by alternative splicing. Mol Pharmacol. 50:799-807.

Cao, Y., M. Nakata, S. Okamoto, E. Takano, T. Yada, Y. Minokoshi, Y. Hirata, K. Nakajima, K. Iskandar, Y. Hayashi, W. Ogawa, G.S. Barsh, H. Hosoda, K. Kangawa, H. Itoh, T. Noda, M. Kasuga, and J. Nakae. 2011. PDK1-Foxo1 in agouti-related peptide neurons regulates energy homeostasis by modulating food intake and energy expenditure. PLoS One. 6:e18324.

Cardone, M.H., N. Roy, H.R. Stennicke, G.S. Salvesen, T.F. Franke, E. Stanbridge, S. Frisch, and J.C. Reed. 1998. Regulation of cell death protease caspase-9 by phosphorylation. Science. 282:1318-1321.

Carpenter, L.R., T.J. Farruggella, A. Symes, M.L. Karow, G.D. Yancopoulos, and N. Stahl. 1998. Enhancing leptin response by preventing SH2-containing phosphatase 2 interaction with Ob receptor. Proc Natl Acad Sci U S A. 95:6061-6066.

Carson, M.J., E.A. Thomas, P.E. Danielson, and J.G. Sutcliffe. 1996. The 5HT5A serotonin receptor is expressed predominantly by astrocytes in which it inhibits cAMP accumulation: a mechanism for neuronal suppression of reactive astrocytes. Glia. 17:317-326.

Caspi, A., K. Sugden, T.E. Moffitt, A. Taylor, I.W. Craig, H. Harrington, J. McClay, J. Mill, J. Martin, A. Braithwaite, and R. Poulton. 2003. Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. Science. 301:386-389.

Chan, S.J., P. Keim, and D.F. Steiner. 1976. Cell-free synthesis of rat preproinsulins: characterization and partial amino acid sequence determination. Proc Natl Acad Sci U S A. 73:1964-1968.

Chaput, Y., P. Blier, and C. de Montigny. 1986. In vivo electrophysiological evidence for the regulatory role of autoreceptors on serotonergic terminals. J Neurosci. 6:2796-2801.

Chen, H., S.J. Wertheimer, C.H. Lin, S.L. Katz, K.E. Amrein, P. Burn, and M.J. Quon. 1997. Protein-tyrosine phosphatases PTP1B and syp are modulators of insulin-stimulated translocation of GLUT4 in transfected rat adipose cells. J Biol Chem. 272:8026-8031.

Chen, L., D.J. Magliano, and P.Z. Zimmet. 2012a. The worldwide epidemiology of type 2 diabetes mellitus--present and future perspectives. Nat Rev Endocrinol. 8:228-236.

Chen, L., G.D. Salinas, and X. Li. 2009. Regulation of serotonin 1B receptor by glycogen synthase kinase-3. Mol Pharmacol. 76:1150-1161.

Chen, S., G.C. Owens, K.L. Crossin, and D.B. Edelman. 2007. Serotonin stimulates mitochondrial transport in hippocampal neurons. Mol Cell Neurosci. 36:472-483.

Chen, X., K.J. Margolis, M.D. Gershon, G.J. Schwartz, and J.Y. Sze. 2012b. Reduced serotonin reuptake transporter (SERT) function causes insulin resistance and hepatic steatosis independent of food intake. PLoS One. 7:e32511.

Chen, Y., B.C. Wang, and Y. Xiao. 2012c. PI3K: a potential therapeutic target for cancer. J Cell Physiol. 227:2818-2821.

Cheng, A., N. Uetani, P.D. Simoncic, V.P. Chaubey, A. Lee-Loy, C.J. McGlade, B.P. Kennedy, and M.L. Tremblay. 2002. Attenuation of leptin action and regulation of obesity by protein tyrosine phosphatase 1B. Dev Cell. 2:497-503.

Ciraolo, E., M. Iezzi, R. Marone, S. Marengo, C. Curcio, C. Costa, O. Azzolino, C. Gonella, C. Rubinetto, H. Wu, W. Dastru, E.L. Martin, L. Silengo, F. Altruda, E. Turco, L. Lanzetti, P. Musiani, T. Ruckle, C. Rommel, J.M. Backer, G. Forni, M.P. Wymann, and E. Hirsch. 2008. Phosphoinositide 3-kinase p110beta activity: key role in metabolism and mammary gland cancer but not development. Sci Signal. 1:ra3.

Clement, S., U. Krause, F. Desmedt, J.F. Tanti, J. Behrends, X. Pesesse, T. Sasaki, J. Penninger, M. Doherty, W. Malaisse, J.E. Dumont, Y. Le Marchand-Brustel, C. Erneux, L. Hue, and S. Schurmans. 2001. The lipid phosphatase SHIP2 controls insulin sensitivity. Nature. 409:92-97.

Clemett, D.A., T. Punhani, M.S. Duxon, T.P. Blackburn, and K.C. Fone. 2000. Immunohistochemical localisation of the 5-HT2C receptor protein in the rat CNS. Neuropharmacology. 39:123-132.

Clodfelder-Miller, B., P. De Sarno, A.A. Zmijewska, L. Song, and R.S. Jope. 2005. Physiological and pathological changes in glucose regulate brain Akt and glycogen synthase kinase-3. J Biol Chem. 280:39723-39731.

Coffer, P.J., and J.R. Woodgett. 1991. Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. Eur J Biochem. 201:475-481.

Coleman, D.L. 1978. Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice. Diabetologia. 14:141-148.

Collin, M., M. Backberg, K. Onnestam, and B. Meister. 2002. 5-HT1A receptor immunoreactivity in hypothalamic neurons involved in body weight control. Neuroreport. 13:945-951.

Cooper, S.J., M.J. Fryer, and J.C. Neill. 1988. Specific effect of putative 5-HT1A agonists, 8-OH-DPAT and gepirone, to increase hypertonic saline consumption in the rat: evidence against a general hyperdipsic action. Physiol Behav. 43:533-537.

Coppari, R., and C. Bjorbaek. 2012. Leptin revisited: its mechanism of action and potential for treating diabetes. Nat Rev Drug Discov. 11:692-708.

Coppen, A. 1967. The biochemistry of affective disorders. Br J Psychiatry. 113:1237-1264.

Copps, K.D., and M.F. White. 2012. Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. Diabetologia. 55:2565-2582.

Cordell, B., G. Bell, E. Tischer, F.M. DeNoto, A. Ullrich, R. Pictet, W.J. Rutter, and H.M. Goodman. 1979. Isolation and characterization of a cloned rat insulin gene. Cell. 18:533-543.

Cowen, D.S. 2007. Serotonin and neuronal growth factors - a convergence of signaling pathways. J Neurochem. 101:1161-1171.

Cowen, D.S., N.N. Johnson-Farley, and T. Travkina. 2005. 5-HT receptors couple to activation of Akt, but not extracellular-regulated kinase (ERK), in cultured hippocampal neurons. J Neurochem. 93:910-917.

Cowen, P.J. 2008. Serotonin and depression: pathophysiological mechanism or marketing myth? Trends Pharmacol Sci. 29:433-436.

Craft, S., and G.S. Watson. 2004. Insulin and neurodegenerative disease: shared and specific mechanisms. Lancet Neurol. 3:169-178.

Cremers, T.I., K. Rea, F.J. Bosker, H.V. Wikstrom, S. Hogg, A. Mork, and B.H. Westerink. 2007. Augmentation of SSRI effects on serotonin by 5-HT2C antagonists: mechanistic studies. Neuropsychopharmacology. 32:1550-1557.

Cross, D.A., D.R. Alessi, P. Cohen, M. Andjelkovich, and B.A. Hemmings. 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature. 378:785-789.

Cryan, J.F., A. Harkin, M. Naughton, J.P. Kelly, and B.E. Leonard. 2000. Characterization of D-fenfluramine-induced hypothermia: evidence for multiple sites of action. Eur J Pharmacol. 390:275-285.

Cuevas, B.D., Y. Lu, M. Mao, J. Zhang, R. LaPushin, K. Siminovitch, and G.B. Mills. 2001. Tyrosine phosphorylation of p85 relieves its inhibitory activity on phosphatidylinositol 3-kinase. J Biol Chem. 276:27455-27461.

Dahlstrom, A., and K. Fuxe. 1964. Localization of monoamines in the lower brain stem. Experientia. 20:398-399.

Damen, J.E., L. Liu, P. Rosten, R.K. Humphries, A.B. Jefferson, P.W. Majerus, and G. Krystal. 1996. The 145-kDa protein induced to associate with Shc by multiple cytokines is an inositol tetraphosphate and phosphatidylinositol 3,4,5-triphosphate 5-phosphatase. Proc Natl Acad Sci U S A. 93:1689-1693.

Datta, S.R., H. Dudek, X. Tao, S. Masters, H. Fu, Y. Gotoh, and M.E. Greenberg. 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell. 91:231-241.

Daws, L.C. 2009. Unfaithful neurotransmitter transporters: focus on serotonin uptake and implications for antidepressant efficacy. Pharmacol Ther. 121:89-99.

De Meyts, P., and J. Whittaker. 2002. Structural biology of insulin and IGF1 receptors: implications for drug design. Nat Rev Drug Discov. 1:769-783.

DeKroon, R., J.B. Robinette, A.B. Hjelmeland, E. Wiggins, M. Blackwell, M. Mihovilovic, M. Fujii, J. York, J. Hart, C. Kontos, J. Rich, and W.J. Strittmatter. 2006. APOE4-VLDL inhibits the HDL-activated phosphatidylinositol 3-kinase/Akt Pathway via the phosphoinositol phosphatase SHIP2. Circ Res. 99:829-836.

Deneris, E.S., and S.C. Wyler. 2012. Serotonergic transcriptional networks and potential importance to mental health. Nat Neurosci. 15:519-527.

Derkach, V., A. Surprenant, and R.A. North. 1989. 5-HT3 receptors are membrane ion channels. Nature. 339:706-709.

Detke, M.J., S. Wieland, and I. Lucki. 1995. Blockade of the antidepressant-like effects of 8-OH-DPAT, buspirone and desipramine in the rat forced swim test by 5HT1A receptor antagonists. Psychopharmacology (Berl). 119:47-54.

Devos, R., Y. Guisez, J. Van der Heyden, D.W. White, M. Kalai, M. Fountoulakis, and G. Plaetinck. 1997. Ligand-independent dimerization of the extracellular domain of the leptin receptor and determination of the stoichiometry of leptin binding. J Biol Chem. 272:18304-18310.

Dhaenen, H. 2001. Imaging the serotonergic system in depression. Eur Arch Psychiatry Clin Neurosci. 251 Suppl 2:II76-80.

Dhand, R., K. Hara, I. Hiles, B. Bax, I. Gout, G. Panayotou, M.J. Fry, K. Yonezawa, M. Kasuga, and M.D. Waterfield. 1994. PI 3-kinase: structural and functional analysis of intersubunit interactions. EMBO J. 13:511-521.

Dhillon, H., J.M. Zigman, C. Ye, C.E. Lee, R.A. McGovern, V. Tang, C.D. Kenny, L.M. Christiansen, R.D. White, E.A. Edelstein, R. Coppari, N. Balthasar, M.A. Cowley, S. Chua, Jr., J.K. Elmquist, and B.B. Lowell.

2006. Leptin directly activates SF1 neurons in the VMH, and this action by leptin is required for normal body-weight homeostasis. Neuron. 49:191-203.

Di Paolo, G., and P. De Camilli. 2006. Phosphoinositides in cell regulation and membrane dynamics. Nature. 443:651-657.

Diaz, S.L., S. Doly, N. Narboux-Neme, S. Fernandez, P. Mazot, S.M. Banas, K. Boutourlinsky, I. Moutkine, A. Belmer, A. Roumier, and L. Maroteaux. 2012. 5-HT(2B) receptors are required for serotonin-selective antidepressant actions. Mol Psychiatry. 17:154-163.

Dimmeler, S., I. Fleming, B. Fisslthaler, C. Hermann, R. Busse, and A.M. Zeiher. 1999. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature. 399:601-605.

DiNitto, J.P., T.C. Cronin, and D.G. Lambright. 2003. Membrane recognition and targeting by lipid-binding domains. Sci STKE. 2003:re16.

Doble, B.W., and J.R. Woodgett. 2003. GSK-3: tricks of the trade for a multi-tasking kinase. J Cell Sci. 116:1175-1186.

Docherty, J.R., and A.R. Green. 2010. The role of monoamines in the changes in body temperature induced by 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) and its derivatives. Br J Pharmacol. 160:1029-1044.

Docherty, K., C.J. Rhodes, N.A. Taylor, K.I. Shennan, and J.C. Hutton. 1989. Proinsulin endopeptidase substrate specificities defined by site-directed mutagenesis of proinsulin. J Biol Chem. 264:18335-18339.

Domin, J., L. Harper, D. Aubyn, M. Wheeler, O. Florey, D. Haskard, M. Yuan, and D. Zicha. 2005. The class II phosphoinositide 3-kinase PI3K-C2beta regulates cell migration by a PtdIns3P dependent mechanism. J Cell Physiol. 205:452-462.

Donovan, M.H., and L.H. Tecott. 2013. Serotonin and the regulation of mammalian energy balance. Front Neurosci. 7:36.

Doucet, E., M. Pohl, C.M. Fattaccini, J. Adrien, S.E. Mestikawy, and M. Hamon. 1995. In situ hybridization evidence for the synthesis of 5-HT1B receptor in serotoninergic neurons of anterior raphe nuclei in the rat brain. Synapse. 19:18-28.

Drong, A.W., C.M. Lindgren, and M.I. McCarthy. 2012. The genetic and epigenetic basis of type 2 diabetes and obesity. Clin Pharmacol Ther. 92:707-715.

Druse, M., N.F. Tajuddin, R.A. Gillespie, and P. Le. 2005. Signaling pathways involved with serotonin1A agonist-mediated neuroprotection against ethanol-induced apoptosis of fetal rhombencephalic neurons. Brain Res Dev Brain Res. 159:18-28.

Duckworth, W.C., R.G. Bennett, and F.G. Hamel. 1998. Insulin degradation: progress and potential. Endocr Rev. 19:608-624.

Dumuis, A., R. Bouhelal, M. Sebben, and J. Bockaert. 1988. A 5-HT receptor in the central nervous system, positively coupled with adenylate cyclase, is antagonized by ICS 205 930. Eur J Pharmacol. 146:187-188.

Durakoglugil, M., A.J. Irving, and J. Harvey. 2005. Leptin induces a novel form of NMDA receptor-dependent long-term depression. J Neurochem. 95:396-405.

Duxon, M.S., T.P. Flanigan, A.C. Reavley, G.S. Baxter, T.P. Blackburn, and K.C. Fone. 1997. Evidence for expression of the 5-hydroxytryptamine-2B receptor protein in the rat central nervous system. Neuroscience. 76:323-329.

Ebina, Y., L. Ellis, K. Jarnagin, M. Edery, L. Graf, E. Clauser, J.H. Ou, F. Masiarz, Y.W. Kan, I.D. Goldfine, and et al. 1985. The human insulin receptor cDNA: the structural basis for hormone-activated transmembrane signalling. Cell. 40:747-758.

Ebrahimkhani, M.R., F. Oakley, L.B. Murphy, J. Mann, A. Moles, M.J. Perugorria, E. Ellis, A.F. Lakey, A.D. Burt, A. Douglass, M.C. Wright, S.A. White, F. Jaffre, L. Maroteaux, and D.A. Mann. 2011. Stimulating healthy tissue regeneration by targeting the 5-HT(2)B receptor in chronic liver disease. Nat Med. 17:1668-1673.

Eck, M.J., S. Dhe-Paganon, T. Trub, R.T. Nolte, and S.E. Shoelson. 1996. Structure of the IRS-1 PTB domain bound to the juxtamembrane region of the insulin receptor. Cell. 85:695-705.

Eckel, R.H., S.E. Kahn, E. Ferrannini, A.B. Goldfine, D.M. Nathan, M.W. Schwartz, R.J. Smith, and S.R. Smith. 2011. Obesity and type 2 diabetes: what can be unified and what needs to be individualized? J Clin Endocrinol Metab. 96:1654-1663.

Elchebly, M., P. Payette, E. Michaliszyn, W. Cromlish, S. Collins, A.L. Loy, D. Normandin, A. Cheng, J. Himms-Hagen, C.C. Chan, C. Ramachandran, M.J. Gresser, M.L. Tremblay, and B.P. Kennedy. 1999. Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. Science. 283:1544-1548.

Emanuelli, B., P. Peraldi, C. Filloux, D. Sawka-Verhelle, D. Hilton, and E. Van Obberghen. 2000. SOCS-3 is an insulin-induced negative regulator of insulin signaling. J Biol Chem. 275:15985-15991.

Embi, N., D.B. Rylatt, and P. Cohen. 1980. Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. Eur J Biochem. 107:519-527.

Engelman, J.A., J. Luo, and L.C. Cantley. 2006. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet. 7:606-619.

Erlander, M.G., T.W. Lovenberg, B.M. Baron, L. de Lecea, P.E. Danielson, M. Racke, A.L. Slone, B.W. Siegel, P.E. Foye, K. Cannon, and et al. 1993. Two members of a distinct subfamily of 5-hydroxytryptamine receptors differentially expressed in rat brain. Proc Natl Acad Sci U S A. 90:3452-3456.

Eskridge, E.M., and D. Shields. 1983. Cell-free processing and segregation of insulin precursors. J Biol Chem. 258:11487-11491.

Facchinetti, V., W. Ouyang, H. Wei, N. Soto, A. Lazorchak, C. Gould, C. Lowry, A.C. Newton, Y. Mao, R.Q. Miao, W.C. Sessa, J. Qin, P. Zhang, B. Su, and E. Jacinto. 2008. The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C. EMBO J. 27:1932-1943.

Faith, M.S., M. Butryn, T.A. Wadden, A. Fabricatore, A.M. Nguyen, and S.B. Heymsfield. 2011. Evidence for prospective associations among depression and obesity in population-based studies. Obes Rev. 12:e438-453.

Falasca, M., W.E. Hughes, V. Dominguez, G. Sala, F. Fostira, M.Q. Fang, R. Cazzolli, P.R. Shepherd, D.E. James, and T. Maffucci. 2007. The role of phosphoinositide 3-kinase C2alpha in insulin signaling. J Biol Chem. 282:28226-28236.

Falasca, M., and T. Maffucci. 2007. Role of class II phosphoinositide 3-kinase in cell signalling. Biochem Soc Trans. 35:211-214.

Falasca, M., and T. Maffucci. 2012. Regulation and cellular functions of class II phosphoinositide 3-kinases. Biochem J. 443:587-601.

Fan, W., D.M. Dinulescu, A.A. Butler, J. Zhou, D.L. Marks, and R.D. Cone. 2000. The central melanocortin system can directly regulate serum insulin levels. Endocrinology. 141:3072-3079.

Fang, X., S. Yu, J.L. Tanyi, Y. Lu, J.R. Woodgett, and G.B. Mills. 2002. Convergence of multiple signaling cascades at glycogen synthase kinase 3: Edg receptor-mediated phosphorylation and inactivation by lysophosphatidic acid through a protein kinase C-dependent intracellular pathway. Mol Cell Biol. 22:2099-2110.

Fantus, I.G. 2011. Insulin resistance and cancer epidemiology, cellular and molecular mechanisms, and clinical implications. In Energy balance and cancer 1. Springer, New York.

Fayard, E., L.A. Tintignac, A. Baudry, and B.A. Hemmings. 2005. Protein kinase B/Akt at a glance. J Cell Sci. 118:5675-5678.

Fayard, E., G. Xue, A. Parcellier, L. Bozulic, and B.A. Hemmings. 2010. Protein kinase B (PKB/Akt), a key mediator of the PI3K signaling pathway. Curr Top Microbiol Immunol. 346:31-56.

Felder, S., M. Zhou, P. Hu, J. Urena, A. Ullrich, M. Chaudhuri, M. White, S.E. Shoelson, and J. Schlessinger. 1993. SH2 domains exhibit high-affinity binding to tyrosine-phosphorylated peptides yet also exhibit rapid dissociation and exchange. Mol Cell Biol. 13:1449-1455.

Feldstein, A., H. Hoagland, K.K. Wong, M.R. Oktem, and H. Freeman. 1964. Mao Activity in Relation to Depression. Am J Psychiatry. 120:1192-1194.

Feng, N., B. Mo, P.L. Johnson, M. Orchinik, C.A. Lowry, and K.J. Renner. 2005. Local inhibition of organic cation transporters increases extracellular serotonin in the medial hypothalamus. Brain Res. 1063:69-76.

Fernandez, S.P., and P. Gaspar. 2012. Investigating anxiety and depressive-like phenotypes in genetic mouse models of serotonin depletion. Neuropharmacology. 62:144-154.

Ferrannini, E., and W.C. Cushman. 2012. Diabetes and hypertension: the bad companions. Lancet. 380:601-610.

Fetalvero, K.M., Y. Yu, M. Goetschkes, G. Liang, R.A. Valdez, T. Gould, E. Triantafellow, S. Bergling, J. Loureiro, J. Eash, V. Lin, J.A. Porter, P.M. Finan, K. Walsh, Y. Yang, X. Mao, and L.O. Murphy. 2013. Defective Autophagy and mTORC1 Signaling in Myotubularin Null Mice. Mol Cell Biol. 33:98-110.

Fetissov, S.O., and M.M. Meguid. 2010. Serotonin delivery into the ventromedial nucleus of the hypothalamus affects differently feeding pattern and body weight in obese and lean Zucker rats. Appetite. 54:346-353.

Finetti, F., M.T. Savino, and C.T. Baldari. 2009. Positive and negative regulation of antigen receptor signaling by the Shc family of protein adapters. Immunol Rev. 232:115-134.

Folli, F., L. Bonfanti, E. Renard, C.R. Kahn, and A. Merighi. 1994. Insulin receptor substrate-1 (IRS-1) distribution in the rat central nervous system. J Neurosci. 14:6412-6422.

Foukas, L.C., C.A. Beeton, J. Jensen, W.A. Phillips, and P.R. Shepherd. 2004. Regulation of phosphoinositide 3-kinase by its intrinsic serine kinase activity in vivo. Mol Cell Biol. 24:966-975.

Foukas, L.C., M. Claret, W. Pearce, K. Okkenhaug, S. Meek, E. Peskett, S. Sancho, A.J. Smith, D.J. Withers, and B. Vanhaesebroeck. 2006. Critical role for the p110alpha phosphoinositide-3-OH kinase in growth and metabolic regulation. Nature. 441:366-370.

Frederich, R.C., A. Hamann, S. Anderson, B. Lollmann, B.B. Lowell, and J.S. Flier. 1995. Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. Nat Med. 1:1311-1314.

Fredriksson, R., M.C. Lagerstrom, L.G. Lundin, and H.B. Schioth. 2003. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol. 63:1256-1272.

Fukuda, M., J.E. Jones, D. Olson, J. Hill, C.E. Lee, L. Gautron, M. Choi, J.M. Zigman, B.B. Lowell, and J.K. Elmquist. 2008. Monitoring FoxO1 localization in chemically identified neurons. J Neurosci. 28:13640-13648.

Fukuda, R., A. Hayashi, A. Utsunomiya, Y. Nukada, R. Fukui, K. Itoh, K. Tezuka, K. Ohashi, K. Mizuno, M. Sakamoto, M. Hamanoue, and T. Tsuji. 2005. Alteration of phosphatidylinositol 3-kinase cascade in the multilobulated nuclear formation of adult T cell leukemia/lymphoma (ATLL). Proc Natl Acad Sci U S A. 102:15213-15218.

Gan, X., J. Wang, B. Su, and D. Wu. 2011. Evidence for direct activation of mTORC2 kinase activity by phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem. 286:10998-11002.

Gao, C., C. Holscher, Y. Liu, and L. Li. 2012. GSK3: a key target for the development of novel treatments for type 2 diabetes mellitus and Alzheimer disease. Rev Neurosci. 23:1-11.

Garber, A.J. 2012. Obesity and type 2 diabetes: which patients are at risk? Diabetes Obes Metab. 14:399-408.

Gardier, A.M., D.J. David, G. Jego, C. Przybylski, C. Jacquot, S. Durier, B. Gruwez, E. Douvier, P. Beauverie, N. Poisson, R. Hen, and M. Bourin. 2003. Effects of chronic paroxetine treatment on dialysate serotonin in 5-HT1B receptor knockout mice. J Neurochem. 86:13-24.

Garza, J.C., M. Guo, W. Zhang, and X.Y. Lu. 2008. Leptin increases adult hippocampal neurogenesis in vivo and in vitro. J Biol Chem. 283:18238-18247.

Garza, J.C., M. Guo, W. Zhang, and X.Y. Lu. 2012. Leptin restores adult hippocampal neurogenesis in a chronic unpredictable stress model of depression and reverses glucocorticoid-induced inhibition of GSK-3beta/beta-catenin signaling. Mol Psychiatry. 17:790-808.

Gaspar, P., O. Cases, and L. Maroteaux. 2003. The developmental role of serotonin: news from mouse molecular genetics. Nat Rev Neurosci. 4:1002-1012.

Gasser, P.J., C.A. Lowry, and M. Orchinik. 2006. Corticosterone-sensitive monoamine transport in the rat dorsomedial hypothalamus: potential role for organic cation transporter 3 in stress-induced modulation of monoaminergic neurotransmission. J Neurosci. 26:8758-8766.

Ge, H., L. Huang, T. Pourbahrami, and C. Li. 2002. Generation of soluble leptin receptor by ectodomain shedding of membrane-spanning receptors in vitro and in vivo. J Biol Chem. 277:45898-45903.

Geering, B., P.R. Cutillas, G. Nock, S.I. Gharbi, and B. Vanhaesebroeck. 2007. Class IA phosphoinositide 3-kinases are obligate p85-p110 heterodimers. Proc Natl Acad Sci U S A. 104:7809-7814.

Gehlert, D.R., D.A. Schober, S.L. Gackenheimer, D.E. Mais, G. Ladouceur, and D.W. Robertson. 1993. Synthesis and evaluation of [125I]-(S)-iodozacopride, a high affinity radioligand for 5HT3 receptors. Neurochem Int. 23:373-383.

Gerard, C., M.P. Martres, K. Lefevre, M.C. Miquel, D. Verge, L. Lanfumey, E. Doucet, M. Hamon, and S. el Mestikawy. 1997. Immuno-localization of serotonin 5-HT6 receptor-like material in the rat central nervous system. Brain Res. 746:207-219.

Gershon, M.D., A.B. Drakontides, and L.L. Ross. 1965. Serotonin: Synthesis and Release from the Myenteric Plexus of the Mouse Intestine. Science. 149:197-199.

Gershon, M.D., and J. Tack. 2007. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology. 132:397-414.

Gewinner, C., Z.C. Wang, A. Richardson, J. Teruya-Feldstein, D. Etemadmoghadam, D. Bowtell, J. Barretina, W.M. Lin, L. Rameh, L. Salmena, P.P. Pandolfi, and L.C. Cantley. 2009. Evidence that inositol polyphosphate 4-phosphatase type II is a tumor suppressor that inhibits PI3K signaling. Cancer Cell. 16:115-125.

Ghilardi, N., and R.C. Skoda. 1997. The leptin receptor activates janus kinase 2 and signals for proliferation in a factor-dependent cell line. Mol Endocrinol. 11:393-399.

Gilbert, F., C.T. Dourish, C. Brazell, S. McClue, and S.M. Stahl. 1988. Relationship of increased food intake and plasma ACTH levels to 5-HT1A receptor activation in rats. Psychoneuroendocrinology. 13:471-478.

Goldstein, B.J., A. Bittner-Kowalczyk, M.F. White, and M. Harbeck. 2000. Tyrosine dephosphorylation and deactivation of insulin receptor substrate-1 by protein-tyrosine phosphatase 1B. Possible facilitation by the formation of a ternary complex with the Grb2 adaptor protein. J Biol Chem. 275:4283-4289.

Gong, Y., R. Ishida-Takahashi, E.C. Villanueva, D.C. Fingar, H. Munzberg, and M.G. Myers, Jr. 2007. The long form of the leptin receptor regulates STAT5 and ribosomal protein S6 via alternate mechanisms. J Biol Chem. 282:31019-31027.

Goodman, H.M. 2009. Basic medical endocrinology. Elsevier/Academic Press, Amsterdam ; Boston. xxxiii, 309 p. pp.

Grayson, B.E., and R.J. Seeley. 2012. Deconstructing obesity: the face of fatness before and after the discovery of leptin. Diabetologia. 55:3-6.

Grayson, B.E., R.J. Seeley, and D.A. Sandoval. 2012. Wired on sugar: the role of the CNS in the regulation of glucose homeostasis. Nat Rev Neurosci.

Grignaschi, G., and R. Samanin. 1992. Role of 5-HT receptors in the effect of d-fenfluramine on feeding patterns in the rat. Eur J Pharmacol. 212:287-289.

Grillo, C.A., G.G. Piroli, K.F. Kaigler, S.P. Wilson, M.A. Wilson, and L.P. Reagan. 2011. Downregulation of hypothalamic insulin receptor expression elicits depressive-like behaviors in rats. Behav Brain Res. 222:230-235.

Grundemann, D., A.C. Koschker, C. Haag, C. Honold, T. Zimmermann, and E. Schomig. 2002. Activation of the extraneuronal monoamine transporter (EMT) from rat expressed in 293 cells. Br J Pharmacol. 137:910-918.

Gunther, L., J. Rothe, A. Rex, J.P. Voigt, M.J. Millan, H. Fink, and B. Bert. 2011. 5-HT(1A)-receptor over-expressing mice: genotype and sex dependent responses to antidepressants in the forced swim-test. Neuropharmacology. 61:433-441.

Guo, K., J.E. McMinn, T. Ludwig, Y.H. Yu, G. Yang, L. Chen, D. Loh, C. Li, S. Chua, Jr., and Y. Zhang. 2007. Disruption of peripheral leptin signaling in mice results in hyperleptinemia without associated metabolic abnormalities. Endocrinology. 148:3987-3997.

Guo, M., T.Y. Huang, J.C. Garza, S.C. Chua, and X.Y. Lu. 2012. Selective deletion of leptin receptors in adult hippocampus induces depression-related behaviours. Int J Neuropsychopharmacol:1-11.

Guo, Z., H. Jiang, X. Xu, W. Duan, and M.P. Mattson. 2008. Leptin-mediated cell survival signaling in hippocampal neurons mediated by JAK STAT3 and mitochondrial stabilization. J Biol Chem. 283:1754-1763.

Guscott, M., L.J. Bristow, K. Hadingham, T.W. Rosahl, M.S. Beer, J.A. Stanton, F. Bromidge, A.P. Owens, I. Huscroft, J. Myers, N.M. Rupniak, S. Patel, P.J. Whiting, P.H. Hutson, K.C. Fone, S.M. Biello, J.J. Kulagowski, and G. McAllister. 2005. Genetic knockout and pharmacological blockade studies of the 5-HT7 receptor suggest therapeutic potential in depression. Neuropharmacology. 48:492-502.

Gustafson, T.A., W. He, A. Craparo, C.D. Schaub, and T.J. O'Neill. 1995. Phosphotyrosine-dependent interaction of SHC and insulin receptor substrate 1 with the NPEY motif of the insulin receptor via a novel non-SH2 domain. Mol Cell Biol. 15:2500-2508.

Hahn, T.M., J.F. Breininger, D.G. Baskin, and M.W. Schwartz. 1998. Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons. Nat Neurosci. 1:271-272.

Hanada, M., J. Feng, and B.A. Hemmings. 2004. Structure, regulation and function of PKB/AKT--a major therapeutic target. Biochim Biophys Acta. 1697:3-16.

Hanger, D.P., B.H. Anderton, and W. Noble. 2009. Tau phosphorylation: the therapeutic challenge for neurodegenerative disease. Trends Mol Med. 15:112-119.

Hargreaves, A.C., S.C. Lummis, and C.W. Taylor. 1994. Ca2+ permeability of cloned and native 5-hydroxytryptamine type 3 receptors. Mol Pharmacol. 46:1120-1128.

Harper, M.E., A. Ullrich, and G.F. Saunders. 1981. Localization of the human insulin gene to the distal end of the short arm of chromosome 11. Proc Natl Acad Sci U S A. 78:4458-4460.

Harwood, H.J., Jr. 2012. The adipocyte as an endocrine organ in the regulation of metabolic homeostasis. Neuropharmacology. 63:57-75.

Hatori, M., C. Vollmers, A. Zarrinpar, L. DiTacchio, E.A. Bushong, S. Gill, M. Leblanc, A. Chaix, M. Joens, J.A. Fitzpatrick, M.H. Ellisman, and S. Panda. 2012. Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet. Cell Metab. 15:848-860.

Havrankova, J., J. Roth, and M. Brownstein. 1978. Insulin receptors are widely distributed in the central nervous system of the rat. Nature. 272:827-829.

Hawkins, P.T., T.R. Jackson, and L.R. Stephens. 1992. Platelet-derived growth factor stimulates synthesis of PtdIns(3,4,5)P3 by activating a PtdIns(4,5)P2 3-OH kinase. Nature. 358:157-159.

Hay, N. 2011. Akt isoforms and glucose homeostasis - the leptin connection. Trends Endocrinol Metab. 22:66-73.

Hayes, D.J., and A.J. Greenshaw. 2011. 5-HT receptors and reward-related behaviour: a review. Neurosci Biobehav Rev. 35:1419-1449.

He, W., A. Craparo, Y. Zhu, T.J. O'Neill, L.M. Wang, J.H. Pierce, and T.A. Gustafson. 1996. Interaction of insulin receptor substrate-2 (IRS-2) with the insulin and insulin-like growth factor I receptors. Evidence for two distinct phosphotyrosine-dependent interaction domains within IRS-2. J Biol Chem. 271:11641-11645.

Hedlund, P.B., and J.G. Sutcliffe. 2004. Functional, molecular and pharmacological advances in 5-HT7 receptor research. Trends Pharmacol Sci. 25:481-486.

Heidenreich, K.A., N.R. Zahniser, P. Berhanu, D. Brandenburg, and J.M. Olefsky. 1983. Structural differences between insulin receptors in the brain and peripheral target tissues. J Biol Chem. 258:8527-8530.

Heisler, L.K., M.A. Cowley, T. Kishi, L.H. Tecott, W. Fan, M.J. Low, J.L. Smart, M. Rubinstein, J. Tatro, J.M. Zigman, R.D. Cone, and J.K. Elmquist. 2003. Central serotonin and melanocortin pathways regulating energy homeostasis. Ann N Y Acad Sci. 994:169-174.

Heisler, L.K., M.A. Cowley, L.H. Tecott, W. Fan, M.J. Low, J.L. Smart, M. Rubinstein, J.B. Tatro, J.N. Marcus, H. Holstege, C.E. Lee, R.D. Cone, and J.K. Elmquist. 2002. Activation of central melanocortin pathways by fenfluramine. Science. 297:609-611.

Heisler, L.K., E.E. Jobst, G.M. Sutton, L. Zhou, E. Borok, Z. Thornton-Jones, H.Y. Liu, J.M. Zigman, N. Balthasar, T. Kishi, C.E. Lee, C.J. Aschkenasi, C.Y. Zhang, J. Yu, O. Boss, K.G. Mountjoy, P.G. Clifton, B.B. Lowell, J.M. Friedman, T. Horvath, A.A. Butler, J.K. Elmquist, and M.A. Cowley. 2006. Serotonin reciprocally regulates melanocortin neurons to modulate food intake. Neuron. 51:239-249.

Herrick-Davis, K., E. Grinde, T.J. Harrigan, and J.E. Mazurkiewicz. 2005. Inhibition of serotonin 5-hydroxytryptamine2c receptor function through heterodimerization: receptor dimers bind two molecules of ligand and one G-protein. J Biol Chem. 280:40144-40151.

Herrick-Davis, K., E. Grinde, T. Lindsley, A. Cowan, and J.E. Mazurkiewicz. 2012. Oligomer size of the serotonin 5-hydroxytryptamine 2C (5-HT2C) receptor revealed by fluorescence correlation spectroscopy with photon counting histogram analysis: evidence for homodimers without monomers or tetramers. J Biol Chem. 287:23604-23614.

Hikiji, K., K. Inoue, S. Iwasaki, K. Ichihara, and N. Kiriike. 2004. Local perfusion of mCPP into ventromedial hypothalamic nucleus, but not into lateral hypothalamic area and frontal cortex, inhibits food intake in rats. Psychopharmacology (Berl). 174:190-196.

Hill, A.J., and J.E. Blundell. 1990. Sensitivity of the appetite control system in obese subjects to nutritional and serotoninergic challenges. Int J Obes. 14:219-233.

Hill, J.W., C.F. Elias, M. Fukuda, K.W. Williams, E.D. Berglund, W.L. Holland, Y.R. Cho, J.C. Chuang, Y. Xu, M. Choi, D. Lauzon, C.E. Lee, R. Coppari, J.A. Richardson, J.M. Zigman, S. Chua, P.E. Scherer, B.B. Lowell, J.C. Bruning, and J.K. Elmquist. 2010. Direct insulin and leptin action on pro-opiomelanocortin neurons is required for normal glucose homeostasis and fertility. Cell Metab. 11:286-297.

Hisadome, K., M.A. Smith, A.I. Choudhury, M. Claret, D.J. Withers, and M.L. Ashford. 2009. 5-HT inhibition of rat insulin 2 promoter Cre recombinase transgene and proopiomelanocortin neuron excitability in the mouse arcuate nucleus. Neuroscience. 159:83-93.

Horn, S., E. Endl, B. Fehse, M.M. Weck, G.W. Mayr, and M. Jucker. 2004. Restoration of SHIP activity in a human leukemia cell line downregulates constitutively activated phosphatidylinositol 3-kinase/Akt/GSK-3beta signaling and leads to an increased transit time through the G1 phase of the cell cycle. Leukemia. 18:1839-1849.

Howard, J.K., B.J. Cave, L.J. Oksanen, I. Tzameli, C. Bjorbaek, and J.S. Flier. 2004. Enhanced leptin sensitivity and attenuation of diet-induced obesity in mice with haploinsufficiency of Socs3. Nat Med. 10:734-738.

Hoyer, D., J.P. Hannon, and G.R. Martin. 2002. Molecular, pharmacological and functional diversity of 5-HT receptors. Pharmacol Biochem Behav. 71:533-554.

Hsieh, J. 2012. Orchestrating transcriptional control of adult neurogenesis. Genes Dev. 26:1010-1021.

Hsiung, S.C., H. Tamir, T.F. Franke, and K.P. Liu. 2005. Roles of extracellular signal-regulated kinase and Akt signaling in coordinating nuclear transcription factor-kappaB-dependent cell survival after serotonin 1A receptor activation. J Neurochem. 95:1653-1666.

Hua, Q. 2010. Insulin: a small protein with a long journey. Protein Cell. 1:537-551.

Huang, G.J., and J. Herbert. 2005. The role of 5-HT1A receptors in the proliferation and survival of progenitor cells in the dentate gyrus of the adult hippocampus and their regulation by corticoids. Neuroscience. 135:803-813.

Huang, J., and B.D. Manning. 2009. A complex interplay between Akt, TSC2 and the two mTOR complexes. Biochem Soc Trans. 37:217-222.

Huang, W., N. Dedousis, B.A. Bhatt, and R.M. O'Doherty. 2004. Impaired activation of phosphatidylinositol 3-kinase by leptin is a novel mechanism of hepatic leptin resistance in diet-induced obesity. J Biol Chem. 279:21695-21700.

Hubbard, S.R. 1997. Crystal structure of the activated insulin receptor tyrosine kinase in complex with peptide substrate and ATP analog. EMBO J. 16:5572-5581.

Hui, L., D.S. Pei, Q.G. Zhang, Q.H. Guan, and G.Y. Zhang. 2005. The neuroprotection of insulin on ischemic brain injury in rat hippocampus through negative regulation of JNK signaling pathway by PI3K/Akt activation. Brain Res. 1052:1-9.

Huszti, Z., and J. Borsy. 1968. Comparative studies on the oxidation of 5-hydroxytryptamine and 3,4-dimethoxyphenylethylamine in rat, dog and human brain. J Neurochem. 15:1407-1413.

Ihle, J.N. 1995. Cytokine receptor signalling. Nature. 377:591-594.

Ikeda, Y., X. Luo, R. Abbud, J.H. Nilson, and K.L. Parker. 1995. The nuclear receptor steroidogenic factor 1 is essential for the formation of the ventromedial hypothalamic nucleus. Mol Endocrinol. 9:478-486.

Ingalls, A.M., M.M. Dickie, and G.D. Snell. 1950. Obese, a new mutation in the house mouse. J Hered. 41:317-318.

Irving, A.J., L. Wallace, D. Durakoglugil, and J. Harvey. 2006. Leptin enhances NR2B-mediated N-methyl-D-aspartate responses via a mitogen-activated protein kinase-dependent process in cerebellar granule cells. Neuroscience. 138:1137-1148.

Iskandar, K., Y. Cao, Y. Hayashi, M. Nakata, E. Takano, T. Yada, C. Zhang, W. Ogawa, M. Oki, S. Chua, Jr., H. Itoh, T. Noda, M. Kasuga, and J. Nakae. 2010. PDK-1/FoxO1 pathway in POMC neurons regulates Pomc expression and food intake. Am J Physiol Endocrinol Metab. 298:E787-798.

Jacobowitz, D.M., and T.L. O'Donohue. 1978. alpha-Melanocyte stimulating hormone: immunohistochemical identification and mapping in neurons of rat brain. Proc Natl Acad Sci U S A. 75:6300-6304.

Jahng, J.W., T.A. Houpt, T.C. Wessel, K. Chen, J.C. Shih, and T.H. Joh. 1997. Localization of monoamine oxidase A and B mRNA in the rat brain by in situ hybridization. Synapse. 25:30-36.

Jefferson, A.B., and P.W. Majerus. 1995. Properties of type II inositol polyphosphate 5-phosphatase. J Biol Chem. 270:9370-9377.

Jiang, L., Z. Li, and L. Rui. 2008. Leptin stimulates both JAK2-dependent and JAK2-independent signaling pathways. J Biol Chem. 283:28066-28073.

Johnson-Farley, N.N., S.B. Kertesy, G.R. Dubyak, and D.S. Cowen. 2005. Enhanced activation of Akt and extracellular-regulated kinase pathways by simultaneous occupancy of Gq-coupled 5-HT2A receptors and Gs-coupled 5-HT7A receptors in PC12 cells. J Neurochem. 92:72-82.

Jones, P.F., T. Jakubowicz, F.J. Pitossi, F. Maurer, and B.A. Hemmings. 1991. Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily. Proc Natl Acad Sci U S A. 88:4171-4175.

Jordan, L.M., J. Liu, P.B. Hedlund, T. Akay, and K.G. Pearson. 2008. Descending command systems for the initiation of locomotion in mammals. Brain Res Rev. 57:183-191.

Jorgensen, S.B., H.M. O'Neill, L. Sylow, J. Honeyman, K.A. Hewitt, R. Palanivel, M.D. Fullerton, L. Oberg, A. Balendran, S. Galic, C. van der Poel, I.A. Trounce, G.S. Lynch, J.D. Schertzer, and G.R. Steinberg. 2013. Deletion of Skeletal Muscle SOCS3 Prevents Insulin Resistance in Obesity. Diabetes. 62:56-64.

Kahn, C.R. 1994. Banting Lecture. Insulin action, diabetogenes, and the cause of type II diabetes. Diabetes. 43:1066-1084.

Kahn, S.E., R.L. Hull, and K.M. Utzschneider. 2006. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature. 444:840-846.

Kanoski, S.E., M.R. Hayes, H.S. Greenwald, S.M. Fortin, C.A. Gianessi, J.R. Gilbert, and H.J. Grill. 2011. Hippocampal leptin signaling reduces food intake and modulates food-related memory processing. Neuropsychopharmacology. 36:1859-1870.

Kastin, A.J., W. Pan, L.M. Maness, R.J. Koletsky, and P. Ernsberger. 1999. Decreased transport of leptin across the blood-brain barrier in rats lacking the short form of the leptin receptor. Peptides. 20:1449-1453.

Kennett, G.A., and G. Curzon. 1991. Potencies of antagonists indicate that 5-HT1C receptors mediate 1-3(chlorophenyl)piperazine-induced hypophagia. Br J Pharmacol. 103:2016-2020.

Kim, B., H.L. Cheng, B. Margolis, and E.L. Feldman. 1998. Insulin receptor substrate 2 and Shc play different roles in insulin-like growth factor I signaling. J Biol Chem. 273:34543-34550.

Kim, B., and E.L. Feldman. 2012. Insulin resistance in the nervous system. Trends Endocrinol Metab. 23:133-141.

Kim, K.W., J. Donato, Jr., E.D. Berglund, Y.H. Choi, D. Kohno, C.F. Elias, R.A. Depinho, and J.K. Elmquist. 2012. FOXO1 in the ventromedial hypothalamus regulates energy balance. J Clin Invest. 122:2578-2589.

Kim, M.S., Y.K. Pak, P.G. Jang, C. Namkoong, Y.S. Choi, J.C. Won, K.S. Kim, S.W. Kim, H.S. Kim, J.Y. Park, Y.B. Kim, and K.U. Lee. 2006. Role of hypothalamic Foxo1 in the regulation of food intake and energy homeostasis. Nat Neurosci. 9:901-906.

Kinchen, J.M., K. Doukoumetzidis, J. Almendinger, L. Stergiou, A. Tosello-Trampont, C.D. Sifri, M.O. Hengartner, and K.S. Ravichandran. 2008. A pathway for phagosome maturation during engulfment of apoptotic cells. Nat Cell Biol. 10:556-566.

Kiser, D., B. Steemers, I. Branchi, and J.R. Homberg. 2012. The reciprocal interaction between serotonin and social behaviour. Neurosci Biobehav Rev. 36:786-798.

Kitano, M., M. Nakaya, T. Nakamura, S. Nagata, and M. Matsuda. 2008. Imaging of Rab5 activity identifies essential regulators for phagosome maturation. Nature. 453:241-245.

Kitchener, S.J., and C.T. Dourish. 1994. An examination of the behavioural specificity of hypophagia induced by 5-HT1B, 5-HT1C and 5-HT2 receptor agonists using the post-prandial satiety sequence in rats. Psychopharmacology (Berl). 113:369-377.

Klockener, T., S. Hess, B.F. Belgardt, L. Paeger, L.A. Verhagen, A. Husch, J.W. Sohn, B. Hampel, H. Dhillon, J.M. Zigman, B.B. Lowell, K.W. Williams, J.K. Elmquist, T.L. Horvath, P. Kloppenburg, and J.C. Bruning. 2011. High-fat feeding promotes obesity via insulin receptor/PI3K-dependent inhibition of SF-1 VMH neurons. Nat Neurosci. 14:911-918.

Knight, Z.A., B. Gonzalez, M.E. Feldman, E.R. Zunder, D.D. Goldenberg, O. Williams, R. Loewith, D. Stokoe, A. Balla, B. Toth, T. Balla, W.A. Weiss, R.L. Williams, and K.M. Shokat. 2006. A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling. Cell. 125:733-747.

Knudsen, L., P. De Meyts, and V.V. Kiselyov. 2011. Insight into the molecular basis for the kinetic differences between the two insulin receptor isoforms. Biochem J. 440:397-403.

Konner, A.C., and J.C. Bruning. 2012. Selective insulin and leptin resistance in metabolic disorders. Cell Metab. 16:144-152.

Konner, A.C., R. Janoschek, L. Plum, S.D. Jordan, E. Rother, X. Ma, C. Xu, P. Enriori, B. Hampel, G.S. Barsh, C.R. Kahn, M.A. Cowley, F.M. Ashcroft, and J.C. Bruning. 2007. Insulin action in AgRP-expressing neurons is required for suppression of hepatic glucose production. Cell Metab. 5:438-449.

Kosofsky, B.E., and M.E. Molliver. 1987. The serotoninergic innervation of cerebral cortex: different classes of axon terminals arise from dorsal and median raphe nuclei. Synapse. 1:153-168.

Kriegsfeld, L.J., and R. Silver. 2006. The regulation of neuroendocrine function: Timing is everything. Horm Behav. 49:557-574.

Kurig, B., A. Shymanets, T. Bohnacker, Prajwal, C. Brock, M.R. Ahmadian, M. Schaefer, A. Gohla, C. Harteneck, M.P. Wymann, E. Jeanclos, and B. Nurnberg. 2009. Ras is an indispensable coregulator of the class IB phosphoinositide 3-kinase p87/p110gamma. Proc Natl Acad Sci U S A. 106:20312-20317.

Lam, D.D., M.J. Przydzial, S.H. Ridley, G.S. Yeo, J.J. Rochford, S. O'Rahilly, and L.K. Heisler. 2008. Serotonin 5-HT2C receptor agonist promotes hypophagia via downstream activation of melanocortin 4 receptors. Endocrinology. 149:1323-1328.

Launay, J.M., P. Herve, K. Peoc'h, C. Tournois, J. Callebert, C.G. Nebigil, N. Etienne, L. Drouet, M. Humbert, G. Simonneau, and L. Maroteaux. 2002. Function of the serotonin 5-hydroxytryptamine 2B receptor in pulmonary hypertension. Nat Med. 8:1129-1135.

Lebrun, P., and E. Van Obberghen. 2008. SOCS proteins causing trouble in insulin action. Acta Physiol (Oxf). 192:29-36.

Lee, D.A., J.L. Bedont, T. Pak, H. Wang, J. Song, A. Miranda-Angulo, V. Takiar, V. Charubhumi, F. Balordi, H. Takebayashi, S. Aja, E. Ford, G. Fishell, and S. Blackshaw. 2012. Tanycytes of the hypothalamic median eminence form a diet-responsive neurogenic niche. Nat Neurosci. 15:700-702.

Lee, G.H., R. Proenca, J.M. Montez, K.M. Carroll, J.G. Darvishzadeh, J.I. Lee, and J.M. Friedman. 1996. Abnormal splicing of the leptin receptor in diabetic mice. Nature. 379:632-635.

Lee, J.O., H. Yang, M.M. Georgescu, A. Di Cristofano, T. Maehama, Y. Shi, J.E. Dixon, P. Pandolfi, and N.P. Pavletich. 1999. Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association. Cell. 99:323-334.

Lee, J.Y., Y.H. Chiu, J. Asara, and L.C. Cantley. 2011. Inhibition of PI3K binding to activators by serine phosphorylation of PI3K regulatory subunit p85alpha Src homology-2 domains. Proc Natl Acad Sci U S A. 108:14157-14162.

Lee, M.J., and S.K. Fried. 2006. Multilevel regulation of leptin storage, turnover, and secretion by feeding and insulin in rat adipose tissue. J Lipid Res. 47:1984-1993.

Lee, M.J., and S.K. Fried. 2009. Integration of hormonal and nutrient signals that regulate leptin synthesis and secretion. Am J Physiol Endocrinol Metab. 296:E1230-1238.

Lee, Y., M.Y. Wang, T. Kakuma, Z.W. Wang, E. Babcock, K. McCorkle, M. Higa, Y.T. Zhou, and R.H. Unger. 2001. Liporegulation in diet-induced obesity. The antisteatotic role of hyperleptinemia. J Biol Chem. 276:5629-5635.

Leibowitz, S.F., and J.T. Alexander. 1998. Hypothalamic serotonin in control of eating behavior, meal size, and body weight. Biol Psychiatry. 44:851-864.

Leibowitz, S.F., G.F. Weiss, and J.S. Suh. 1990. Medial hypothalamic nuclei mediate serotonin's inhibitory effect on feeding behavior. Pharmacol Biochem Behav. 37:735-742.

Leinninger, G.M., D.M. Opland, Y.H. Jo, M. Faouzi, L. Christensen, L.A. Cappellucci, C.J. Rhodes, M.E. Gnegy, J.B. Becker, E.N. Pothos, A.F. Seasholtz, R.C. Thompson, and M.G. Myers, Jr. 2011. Leptin action

via neurotensin neurons controls orexin, the mesolimbic dopamine system and energy balance. Cell Metab. 14:313-323.

Leone, A.M., M. Errico, S.L. Lin, and D.S. Cowen. 2000. Activation of extracellular signal-regulated kinase (ERK) and Akt by human serotonin 5-HT(1B) receptors in transfected BE(2)-C neuroblastoma cells is inhibited by RGS4. J Neurochem. 75:934-938.

Lesch, K.P., and J. Waider. 2012. Serotonin in the modulation of neural plasticity and networks: implications for neurodevelopmental disorders. Neuron. 76:175-191.

Leshan, R.L., M. Greenwald-Yarnell, C.M. Patterson, I.E. Gonzalez, and M.G. Myers, Jr. 2012. Leptin action through hypothalamic nitric oxide synthase-1-expressing neurons controls energy balance. Nat Med. 18:820-823.

Leto, D., and A.R. Saltiel. 2012. Regulation of glucose transport by insulin: traffic control of GLUT4. Nat Rev Mol Cell Biol. 13:383-396.

Levkovitz, Y., G. Ben-Shushan, A. Hershkovitz, R. Isaac, I. Gil-Ad, D. Shvartsman, D. Ronen, A. Weizman, and Y. Zick. 2007. Antidepressants induce cellular insulin resistance by activation of IRS-1 kinases. Mol Cell Neurosci. 36:305-312.

Li, J., C. Yen, D. Liaw, K. Podsypanina, S. Bose, S.I. Wang, J. Puc, C. Miliaresis, L. Rodgers, R. McCombie, S.H. Bigner, B.C. Giovanella, M. Ittmann, B. Tycko, H. Hibshoosh, M.H. Wigler, and R. Parsons. 1997. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science. 275:1943-1947.

Li, J.Y., S. Finniss, Y.K. Yang, Q. Zeng, S.Y. Qu, G. Barsh, C. Dickinson, and I. Gantz. 2000. Agouti-related protein-like immunoreactivity: characterization of release from hypothalamic tissue and presence in serum. Endocrinology. 141:1942-1950.

Li, Q., T. Hosaka, Y. Shikama, Y. Bando, C. Kosugi, N. Kataoka, Y. Nakaya, and M. Funaki. 2012. Heparin-binding EGF-like growth factor (HB-EGF) mediates 5-HT-induced insulin resistance through activation of EGF receptor-ERK1/2-mTOR pathway. Endocrinology. 153:56-68.

Li, Q.H., K. Nakadate, S. Tanaka-Nakadate, D. Nakatsuka, Y. Cui, and Y. Watanabe. 2004a. Unique expression patterns of 5-HT2A and 5-HT2C receptors in the rat brain during postnatal development: Western blot and immunohistochemical analyses. J Comp Neurol. 469:128-140.

Li, X., and R.S. Jope. 2010. Is glycogen synthase kinase-3 a central modulator in mood regulation? Neuropsychopharmacology. 35:2143-2154.

Li, X., K.M. Rosborough, A.B. Friedman, W. Zhu, and K.A. Roth. 2007a. Regulation of mouse brain glycogen synthase kinase-3 by atypical antipsychotics. Int J Neuropsychopharmacol. 10:7-19.

Li, X., W. Zhu, M.S. Roh, A.B. Friedman, K. Rosborough, and R.S. Jope. 2004b. In vivo regulation of glycogen synthase kinase-3beta (GSK3beta) by serotonergic activity in mouse brain. Neuropsychopharmacology. 29:1426-1431.

Li, X.L., S. Aou, Y. Oomura, N. Hori, K. Fukunaga, and T. Hori. 2002. Impairment of long-term potentiation and spatial memory in leptin receptor-deficient rodents. Neuroscience. 113:607-615.

Li, Z., Y. Zhou, C. Carter-Su, M.G. Myers, Jr., and L. Rui. 2007b. SH2B1 enhances leptin signaling by both Janus kinase 2 Tyr813 phosphorylation-dependent and -independent mechanisms. Mol Endocrinol. 21:2270-2281.

Liang, C., P. Feng, B. Ku, I. Dotan, D. Canaani, B.H. Oh, and J.U. Jung. 2006. Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. Nat Cell Biol. 8:688-699.

Liberman, Z., and H. Eldar-Finkelman. 2005. Serine 332 phosphorylation of insulin receptor substrate-1 by glycogen synthase kinase-3 attenuates insulin signaling. J Biol Chem. 280:4422-4428.

Lin, H.V., L. Plum, H. Ono, R. Gutierrez-Juarez, M. Shanabrough, E. Borok, T.L. Horvath, L. Rossetti, and D. Accili. 2010. Divergent regulation of energy expenditure and hepatic glucose production by insulin receptor in agouti-related protein and POMC neurons. Diabetes. 59:337-346.

Loh, K., A. Fukushima, X. Zhang, S. Galic, D. Briggs, P.J. Enriori, S. Simonds, F. Wiede, A. Reichenbach, C. Hauser, N.A. Sims, K.K. Bence, S. Zhang, Z.Y. Zhang, B.B. Kahn, B.G. Neel, Z.B. Andrews, M.A. Cowley, and T. Tiganis. 2011. Elevated hypothalamic TCPTP in obesity contributes to cellular leptin resistance. Cell Metab. 14:684-699.

Lowe, W.L., Jr., F.T. Boyd, D.W. Clarke, M.K. Raizada, C. Hart, and D. LeRoith. 1986. Development of brain insulin receptors: structural and functional studies of insulin receptors from whole brain and primary cell cultures. Endocrinology. 119:25-35.

Lu, D., D. Willard, I.R. Patel, S. Kadwell, L. Overton, T. Kost, M. Luther, W. Chen, R.P. Woychik, W.O. Wilkison, and et al. 1994. Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. Nature. 371:799-802.

Lu, X.Y. 2007. The leptin hypothesis of depression: a potential link between mood disorders and obesity? Curr Opin Pharmacol. 7:648-652.

Lu, X.Y., C.S. Kim, A. Frazer, and W. Zhang. 2006. Leptin: a potential novel antidepressant. Proc Natl Acad Sci U S A. 103:1593-1598.

Lucas, G., V.V. Rymar, J. Du, O. Mnie-Filali, C. Bisgaard, S. Manta, L. Lambas-Senas, O. Wiborg, N. Haddjeri, G. Pineyro, A.F. Sadikot, and G. Debonnel. 2007. Serotonin(4) (5-HT(4)) receptor agonists are putative antidepressants with a rapid onset of action. Neuron. 55:712-725.

Lucas, J.J., A. Yamamoto, K. Scearce-Levie, F. Saudou, and R. Hen. 1998. Absence of fenfluramine-induced anorexia and reduced c-Fos induction in the hypothalamus and central amygdaloid complex of serotonin 1B receptor knock-out mice. J Neurosci. 18:5537-5544.

Lummis, S.C. 2012. 5-HT(3) receptors. J Biol Chem. 287:40239-40245.

Lund, I.K., J.A. Hansen, H.S. Andersen, N.P. Moller, and N. Billestrup. 2005. Mechanism of protein tyrosine phosphatase 1B-mediated inhibition of leptin signalling. J Mol Endocrinol. 34:339-351.

Ma, K., S.M. Cheung, A.J. Marshall, and V. Duronio. 2008. PI(3,4,5)P3 and PI(3,4)P2 levels correlate with PKB/akt phosphorylation at Thr308 and Ser473, respectively; PI(3,4)P2 levels determine PKB activity. Cell Signal. 20:684-694.

Maehama, T., and J.E. Dixon. 1998. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem. 273:13375-13378.

Maier, U., A. Babich, and B. Nurnberg. 1999. Roles of non-catalytic subunits in gbetagamma-induced activation of class I phosphoinositide 3-kinase isoforms beta and gamma. J Biol Chem. 274:29311-29317.

Malagie, I., D.J. David, P. Jolliet, R. Hen, M. Bourin, and A.M. Gardier. 2002. Improved efficacy of fluoxetine in increasing hippocampal 5-hydroxytryptamine outflow in 5-HT(1B) receptor knock-out mice. Eur J Pharmacol. 443:99-104.

Malik, V.S., W.C. Willett, and F.B. Hu. 2013. Global obesity: trends, risk factors and policy implications. Nat Rev Endocrinol. 9:13-27.

Malone, H.M., J.A. Peters, and J.J. Lambert. 1991. Physiological and pharmacological properties of 5-HT3 receptors--a patch clamp-study. Neuropeptides. 19 Suppl:25-30.

Mamounas, L.A., C.A. Mullen, E. O'Hearn, and M.E. Molliver. 1991. Dual serotoninergic projections to forebrain in the rat: morphologically distinct 5-HT axon terminals exhibit differential vulnerability to neurotoxic amphetamine derivatives. J Comp Neurol. 314:558-586.

Man, H.Y., J.W. Lin, W.H. Ju, G. Ahmadian, L. Liu, L.E. Becker, M. Sheng, and Y.T. Wang. 2000. Regulation of AMPA receptor-mediated synaptic transmission by clathrin-dependent receptor internalization. Neuron. 25:649-662.

Mancour, L.V., H.N. Daghestani, S. Dutta, G.H. Westfield, J. Schilling, A.N. Oleskie, J.F. Herbstman, S.Z. Chou, and G. Skiniotis. 2012. Ligand-induced architecture of the leptin receptor signaling complex. Mol Cell. 48:655-661.

Manning, B.D., and L.C. Cantley. 2007. AKT/PKB signaling: navigating downstream. Cell. 129:1261-1274.

Margetic, S., C. Gazzola, G.G. Pegg, and R.A. Hill. 2002. Leptin: a review of its peripheral actions and interactions. Int J Obes Relat Metab Disord. 26:1407-1433.

Marks, J.L., D. Porte, Jr., W.L. Stahl, and D.G. Baskin. 1990. Localization of insulin receptor mRNA in rat brain by in situ hybridization. Endocrinology. 127:3234-3236.

Marroqui, L., A. Gonzalez, P. Neco, E. Caballero-Garrido, E. Vieira, C. Ripoll, A. Nadal, and I. Quesada. 2012. Role of leptin in the pancreatic beta-cell: effects and signaling pathways. J Mol Endocrinol. 49:R9-17.

Martin, E.D., A. Sanchez-Perez, J.L. Trejo, J.A. Martin-Aldana, M. Cano Jaimez, S. Pons, C. Acosta Umanzor, L. Menes, M.F. White, and D.J. Burks. 2012. IRS-2 Deficiency impairs NMDA receptor-dependent long-term potentiation. Cereb Cortex. 22:1717-1727.

Matsunaga, K., T. Saitoh, K. Tabata, H. Omori, T. Satoh, N. Kurotori, I. Maejima, K. Shirahama-Noda, T. Ichimura, T. Isobe, S. Akira, T. Noda, and T. Yoshimori. 2009. Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. Nat Cell Biol. 11:385-396.

Matthes, H., U. Boschert, N. Amlaiky, R. Grailhe, J.L. Plassat, F. Muscatelli, M.G. Mattei, and R. Hen. 1993. Mouse 5-hydroxytryptamine5A and 5-hydroxytryptamine5B receptors define a new family of serotonin receptors: cloning, functional expression, and chromosomal localization. Mol Pharmacol. 43:313-319.

Maures, T.J., J.H. Kurzer, and C. Carter-Su. 2007. SH2B1 (SH2-B) and JAK2: a multifunctional adaptor protein and kinase made for each other. Trends Endocrinol Metab. 18:38-45.

Mayo, L.D., and D.B. Donner. 2001. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. Proc Natl Acad Sci U S A. 98:11598-11603.

McGlade, C.J., C. Ellis, M. Reedijk, D. Anderson, G. Mbamalu, A.D. Reith, G. Panayotou, P. End, A. Bernstein, A. Kazlauskas, and et al. 1992. SH2 domains of the p85 alpha subunit of phosphatidylinositol 3-kinase regulate binding to growth factor receptors. Mol Cell Biol. 12:991-997.

Mehran, A.E., N.M. Templeman, G.S. Brigidi, G.E. Lim, K.Y. Chu, X. Hu, J.D. Botezelli, A. Asadi, B.G. Hoffman, T.J. Kieffer, S.X. Bamji, S.M. Clee, and J.D. Johnson. 2012. Hyperinsulinemia drives diet-induced obesity independently of brain insulin production. Cell Metab. 16:723-737.

Mendels, J. 1987. Clinical experience with serotonin reuptake inhibiting antidepressants. J Clin Psychiatry. 48 Suppl:26-30.

Menting, J.G., J. Whittaker, M.B. Margetts, L.J. Whittaker, G.K. Kong, B.J. Smith, C.J. Watson, L. Zakova, E. Kletvikova, J. Jiracek, S.J. Chan, D.F. Steiner, G.G. Dodson, A.M. Brzozowski, M.A. Weiss, C.W. Ward, and M.C. Lawrence. 2013. How insulin engages its primary binding site on the insulin receptor. Nature. 493:241-245.

Milburn, C.C., M. Deak, S.M. Kelly, N.C. Price, D.R. Alessi, and D.M. Van Aalten. 2003. Binding of phosphatidylinositol 3,4,5-trisphosphate to the pleckstrin homology domain of protein kinase B induces a conformational change. Biochem J. 375:531-538.

Millan, M.J., P. Marin, J. Bockaert, and C. Mannoury la Cour. 2008. Signaling at G-protein-coupled serotonin receptors: recent advances and future research directions. Trends Pharmacol Sci. 29:454-464.

Miller, J.M., K.G. Brennan, T.R. Ogden, M.A. Oquendo, G.M. Sullivan, J.J. Mann, and R.V. Parsey. 2009. Elevated serotonin 1A binding in remitted major depressive disorder: evidence for a trait biological abnormality. Neuropsychopharmacology. 34:2275-2284.

Minokoshi, Y., Y.B. Kim, O.D. Peroni, L.G. Fryer, C. Muller, D. Carling, and B.B. Kahn. 2002. Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. Nature. 415:339-343.

Mintun, M.A., Y.I. Sheline, S.M. Moerlein, A.G. Vlassenko, Y. Huang, and A.Z. Snyder. 2004. Decreased hippocampal 5-HT2A receptor binding in major depressive disorder: in vivo measurement with [18F]altanserin positron emission tomography. Biol Psychiatry. 55:217-224.

Miyata, S., S. Hirano, and J. Kamei. 2004. Diabetes attenuates the antidepressant-like effect mediated by the activation of 5-HT1A receptor in the mouse tail suspension test. Neuropsychopharmacology. 29:461-469.

Mohammad-Qureshi, S.S., M.D. Jennings, and G.D. Pavitt. 2008. Clues to the mechanism of action of eIF2B, the guanine-nucleotide-exchange factor for translation initiation. Biochem Soc Trans. 36:658-664.

Mokler, D.J., J.D. Bronzino, J.R. Galler, and P.J. Morgane. 1999. The effects of median raphe electrical stimulation on serotonin release in the dorsal hippocampal formation of prenatally protein malnourished rats. Brain Res. 838:95-103.

Mombereau, C., Y. Kawahara, B.B. Gundersen, K. Nishikura, and J.A. Blendy. 2010. Functional relevance of serotonin 2C receptor mRNA editing in antidepressant- and anxiety-like behaviors. Neuropharmacology. 59:468-473.

Monti, J.M. 2011. Serotonin control of sleep-wake behavior. Sleep Med Rev. 15:269-281.

Mori, H., R. Hanada, T. Hanada, D. Aki, R. Mashima, H. Nishinakamura, T. Torisu, K.R. Chien, H. Yasukawa, and A. Yoshimura. 2004. Socs3 deficiency in the brain elevates leptin sensitivity and confers resistance to diet-induced obesity. Nat Med. 10:739-743.

Morioka, T., E. Asilmaz, J. Hu, J.F. Dishinger, A.J. Kurpad, C.F. Elias, H. Li, J.K. Elmquist, R.T. Kennedy, and R.N. Kulkarni. 2007. Disruption of leptin receptor expression in the pancreas directly affects beta cell growth and function in mice. J Clin Invest. 117:2860-2868.

Moriya, T., Y. Yoshinobu, M. Ikeda, S. Yokota, M. Akiyama, and S. Shibata. 1998. Potentiating action of MKC-242, a selective 5-HT1A receptor agonist, on the photic entrainment of the circadian activity rhythm in hamsters. Br J Pharmacol. 125:1281-1287.

Morris, D.L., and L. Rui. 2009. Recent advances in understanding leptin signaling and leptin resistance. Am J Physiol Endocrinol Metab. 297:E1247-1259.

Morrison, C.D. 2009. Leptin signaling in brain: A link between nutrition and cognition? Biochim Biophys Acta. 1792:401-408.

Moult, P.R., A. Cross, S.D. Santos, A.L. Carvalho, Y. Lindsay, C.N. Connolly, A.J. Irving, N.R. Leslie, and J. Harvey. 2010. Leptin regulates AMPA receptor trafficking via PTEN inhibition. J Neurosci. 30:4088-4101.

Moult, P.R., B. Milojkovic, and J. Harvey. 2009. Leptin reverses long-term potentiation at hippocampal CA1 synapses. J Neurochem. 108:685-696.

Mountjoy, K.G. 2010. Functions for pro-opiomelanocortin-derived peptides in obesity and diabetes. Biochem J. 428:305-324.

Mountjoy, K.G., M.T. Mortrud, M.J. Low, R.B. Simerly, and R.D. Cone. 1994. Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. Mol Endocrinol. 8:1298-1308.

Müller, C.P., and B.L. Jacobs. 2010. Handbook of the behavioral neurobiology of serotonin. In Handbook of behavioral neuroscience v 18. Elsevier/Academic Press, Amsterdam.

Mullins, D., D. Kirby, J. Hwa, M. Guzzi, J. Rivier, and E. Parker. 2001. Identification of potent and selective neuropeptide Y Y(1) receptor agonists with orexigenic activity in vivo. Mol Pharmacol. 60:534-540.

Munday, A.D., F.A. Norris, K.K. Caldwell, S. Brown, P.W. Majerus, and C.A. Mitchell. 1999. The inositol polyphosphate 4-phosphatase forms a complex with phosphatidylinositol 3-kinase in human platelet cytosol. Proc Natl Acad Sci U S A. 96:3640-3645.

Myers, M.G., Jr., J.M. Backer, X.J. Sun, S. Shoelson, P. Hu, J. Schlessinger, M. Yoakim, B. Schaffhausen, and M.F. White. 1992. IRS-1 activates phosphatidylinositol 3'-kinase by associating with src homology 2 domains of p85. Proc Natl Acad Sci U S A. 89:10350-10354.

Myers, M.G., Jr., T.C. Grammer, J. Brooks, E.M. Glasheen, L.M. Wang, X.J. Sun, J. Blenis, J.H. Pierce, and M.F. White. 1995. The pleckstrin homology domain in insulin receptor substrate-1 sensitizes insulin signaling. J Biol Chem. 270:11715-11718.

Myers, M.G., Jr., S.B. Heymsfield, C. Haft, B.B. Kahn, M. Laughlin, R.L. Leibel, M.H. Tschop, and J.A. Yanovski. 2012. Challenges and opportunities of defining clinical leptin resistance. Cell Metab. 15:150-156.

Myers, M.G., Jr., R.L. Leibel, R.J. Seeley, and M.W. Schwartz. 2010. Obesity and leptin resistance: distinguishing cause from effect. Trends Endocrinol Metab. 21:643-651.

Myers, M.G., Jr., L.M. Wang, X.J. Sun, Y. Zhang, L. Yenush, J. Schlessinger, J.H. Pierce, and M.F. White. 1994. Role of IRS-1-GRB-2 complexes in insulin signaling. Mol Cell Biol. 14:3577-3587.

Myers, M.P., J.P. Stolarov, C. Eng, J. Li, S.I. Wang, M.H. Wigler, R. Parsons, and N.K. Tonks. 1997. P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. Proc Natl Acad Sci U S A. 94:9052-9057.

Naggert, J.K., L.D. Fricker, O. Varlamov, P.M. Nishina, Y. Rouille, D.F. Steiner, R.J. Carroll, B.J. Paigen, and E.H. Leiter. 1995. Hyperproinsulinaemia in obese fat/fat mice associated with a carboxypeptidase E mutation which reduces enzyme activity. Nat Genet. 10:135-142.

Neel, B.G., H. Gu, and L. Pao. 2003. The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. Trends Biochem Sci. 28:284-293.

Neumaier, J.F., T.J. Sexton, J. Yracheta, A.M. Diaz, and M. Brownfield. 2001. Localization of 5-HT(7) receptors in rat brain by immunocytochemistry, in situ hybridization, and agonist stimulated cFos expression. J Chem Neuroanat. 21:63-73.

Nikiforuk, A., T. Kos, and A. Wesolowska. 2011. The 5-HT6 receptor agonist EMD 386088 produces antidepressant and anxiolytic effects in rats after intrahippocampal administration. Psychopharmacology (Berl). 217:411-418.

Nonogaki, K., A.M. Strack, M.F. Dallman, and L.H. Tecott. 1998. Leptin-independent hyperphagia and type 2 diabetes in mice with a mutated serotonin 5-HT2C receptor gene. Nat Med. 4:1152-1156.

Nouwen, A., K. Winkley, J. Twisk, C.E. Lloyd, M. Peyrot, K. Ismail, and F. Pouwer. 2010. Type 2 diabetes mellitus as a risk factor for the onset of depression: a systematic review and meta-analysis. Diabetologia. 53:2480-2486.

O'Malley, D., A.J. Irving, and J. Harvey. 2005. Leptin-induced dynamic alterations in the actin cytoskeleton mediate the activation and synaptic clustering of BK channels. FASEB J. 19:1917-1919.

O'Neill, T.J., A. Craparo, and T.A. Gustafson. 1994. Characterization of an interaction between insulin receptor substrate 1 and the insulin receptor by using the two-hybrid system. Mol Cell Biol. 14:6433-6442.

Obici, S., Z. Feng, G. Karkanias, D.G. Baskin, and L. Rossetti. 2002a. Decreasing hypothalamic insulin receptors causes hyperphagia and insulin resistance in rats. Nat Neurosci. 5:566-572.

Obici, S., B.B. Zhang, G. Karkanias, and L. Rossetti. 2002b. Hypothalamic insulin signaling is required for inhibition of glucose production. Nat Med. 8:1376-1382.

Okada, S., M. Matsuda, M. Anafi, T. Pawson, and J.E. Pessin. 1998. Insulin regulates the dynamic balance between Ras and Rap1 signaling by coordinating the assembly states of the Grb2-SOS and CrkII-C3G complexes. EMBO J. 17:2554-2565.

Oliver, K.R., A.M. Kinsey, A. Wainwright, and D.J. Sirinathsinghji. 2000. Localization of 5-ht(5A) receptor-like immunoreactivity in the rat brain. Brain Res. 867:131-142.

Ollmann, M.M., B.D. Wilson, Y.K. Yang, J.A. Kerns, Y. Chen, I. Gantz, and G.S. Barsh. 1997. Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. Science. 278:135-138.

Ooms, L.M., K.A. Horan, P. Rahman, G. Seaton, R. Gurung, D.S. Kethesparan, and C.A. Mitchell. 2009. The role of the inositol polyphosphate 5-phosphatases in cellular function and human disease. Biochem J. 419:29-49.

Oomura, Y., N. Hori, T. Shiraishi, K. Fukunaga, H. Takeda, M. Tsuji, T. Matsumiya, M. Ishibashi, S. Aou, X.L. Li, D. Kohno, K. Uramura, H. Sougawa, T. Yada, M.J. Wayner, and K. Sasaki. 2006. Leptin facilitates learning and memory performance and enhances hippocampal CA1 long-term potentiation and CaMK II phosphorylation in rats. Peptides. 27:2738-2749.

Owashi, T., S. Iritani, K. Niizato, K. Ikeda, and K. Kamijima. 2004. The distribution of serotonin transporter immunoreactivity in hippocampal formation in monkeys and rats. Brain Res. 1010:166-168.

Ozes, O.N., L.D. Mayo, J.A. Gustin, S.R. Pfeffer, L.M. Pfeffer, and D.B. Donner. 1999. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature. 401:82-85.

Paez, X., and S.F. Leibowitz. 1993. Changes in extracellular PVN monoamines and macronutrient intake after idazoxan or fluoxetine injection. Pharmacol Biochem Behav. 46:933-941.

Palovcik, R.A., M.I. Phillips, M.S. Kappy, and M.K. Raizada. 1984. Insulin inhibits pyramidal neurons in hippocampal slices. Brain Res. 309:187-191.

Pan, A., N. Keum, O.I. Okereke, Q. Sun, M. Kivimaki, R.R. Rubin, and F.B. Hu. 2012. Bidirectional association between depression and metabolic syndrome: a systematic review and meta-analysis of epidemiological studies. Diabetes Care. 35:1171-1180.

Pan, W., H. Hsuchou, H. Tu, and A.J. Kastin. 2008. Developmental changes of leptin receptors in cerebral microvessels: unexpected relation to leptin transport. Endocrinology. 149:877-885.

Papakostas, G.I., M.E. Thase, M. Fava, J.C. Nelson, and R.C. Shelton. 2007. Are antidepressant drugs that combine serotonergic and noradrenergic mechanisms of action more effective than the selective serotonin reuptake inhibitors in treating major depressive disorder? A meta-analysis of studies of newer agents. Biol Psychiatry. 62:1217-1227.

Papas, M.A., A.J. Alberg, R. Ewing, K.J. Helzlsouer, T.L. Gary, and A.C. Klassen. 2007. The built environment and obesity. Epidemiol Rev. 29:129-143.

Papazoglou, I., F. Berthou, N. Vicaire, C. Rouch, E.M. Markaki, D. Bailbe, B. Portha, M. Taouis, and K. Gerozissis. 2012. Hypothalamic serotonin-insulin signaling cross-talk and alterations in a type 2 diabetic model. Mol Cell Endocrinol. 350:136-144.

Pascual-Brazo, J., E. Castro, A. Diaz, E.M. Valdizan, F. Pilar-Cuellar, R. Vidal, B. Treceno, and A. Pazos. 2012. Modulation of neuroplasticity pathways and antidepressant-like behavioural responses following the short-term (3 and 7 days) administration of the 5-HT(4) receptor agonist RS67333. Int J Neuropsychopharmacol. 15:631-643.

Patterson, C.M., R.L. Leshan, J.C. Jones, and M.G. Myers, Jr. 2011. Molecular mapping of mouse brain regions innervated by leptin receptor-expressing cells. Brain Res. 1378:18-28.

Paxinos, G., and C. Watson. 2005. The rat brain in stereotaxic coordinates. Elsevier Academic Press, Amsterdam ; Boston. xliii, 166 p. pp.

Pazos, A., and J.M. Palacios. 1985. Quantitative autoradiographic mapping of serotonin receptors in the rat brain. I. Serotonin-1 receptors. Brain Res. 346:205-230.

Pedrazzini, T., J. Seydoux, P. Kunstner, J.F. Aubert, E. Grouzmann, F. Beermann, and H.R. Brunner. 1998. Cardiovascular response, feeding behavior and locomotor activity in mice lacking the NPY Y1 receptor. Nat Med. 4:722-726.

Pelicci, G., L. Lanfrancone, F. Grignani, J. McGlade, F. Cavallo, G. Forni, I. Nicoletti, T. Pawson, and P.G. Pelicci. 1992. A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. Cell. 70:93-104.

Penington, N.J., J.S. Kelly, and A.P. Fox. 1993. Whole-cell recordings of inwardly rectifying K+ currents activated by 5-HT1A receptors on dorsal raphe neurones of the adult rat. J Physiol. 469:387-405.

Peraldi, P., C. Filloux, B. Emanuelli, D.J. Hilton, and E. Van Obberghen. 2001. Insulin induces suppressor of cytokine signaling-3 tyrosine phosphorylation through janus-activated kinase. J Biol Chem. 276:24614-24620.

Perino, A., A. Ghigo, E. Ferrero, F. Morello, G. Santulli, G.S. Baillie, F. Damilano, A.J. Dunlop, C. Pawson, R. Walser, R. Levi, F. Altruda, L. Silengo, L.K. Langeberg, G. Neubauer, S. Heymans, G. Lembo, M.P. Wymann, R. Wetzker, M.D. Houslay, G. Iaccarino, J.D. Scott, and E. Hirsch. 2011. Integrating cardiac PIP3 and cAMP signaling through a PKA anchoring function of p110gamma. Mol Cell. 42:84-95.

Pesesse, X., C. Moreau, A.L. Drayer, R. Woscholski, P. Parker, and C. Erneux. 1998. The SH2 domain containing inositol 5-phosphatase SHIP2 displays phosphatidylinositol 3,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate 5-phosphatase activity. FEBS Lett. 437:301-303.

Picarel-Blanchot, F., D. Bailbe, and B. Portha. 1994. d-Fenfluramine improves hepatic insulin action in streptozotocin-diabetic rats. Eur J Pharmacol. 264:227-232.

Plassat, J.L., N. Amlaiky, and R. Hen. 1993. Molecular cloning of a mammalian serotonin receptor that activates adenylate cyclase. Mol Pharmacol. 44:229-236.

Plum, L., X. Ma, B. Hampel, N. Balthasar, R. Coppari, H. Munzberg, M. Shanabrough, D. Burdakov, E. Rother, R. Janoschek, J. Alber, B.F. Belgardt, L. Koch, J. Seibler, F. Schwenk, C. Fekete, A. Suzuki, T.W. Mak, W. Krone, T.L. Horvath, F.M. Ashcroft, and J.C. Bruning. 2006. Enhanced PIP3 signaling in POMC neurons causes KATP channel activation and leads to diet-sensitive obesity. J Clin Invest. 116:1886-1901.

Polter, A., S. Yang, A.A. Zmijewska, T. van Groen, J.H. Paik, R.A. Depinho, S.L. Peng, R.S. Jope, and X. Li. 2009. Forkhead box, class O transcription factors in brain: regulation and behavioral manifestation. Biol Psychiatry. 65:150-159.

Polter, A.M., and X. Li. 2010. 5-HT1A receptor-regulated signal transduction pathways in brain. Cell Signal. 22:1406-1412.

Polter, A.M., and X. Li. 2011. Glycogen Synthase Kinase-3 is an Intermediate Modulator of Serotonin Neurotransmission. Front Mol Neurosci. 4:31.

Polter, A.M., S. Yang, R.S. Jope, and X. Li. 2012. Functional significance of glycogen synthase kinase-3 regulation by serotonin. Cell Signal. 24:265-271.

Pugliese, A.M., M.B. Passani, and R. Corradetti. 1998. Effect of the selective 5-HT1A receptor antagonist WAY 100635 on the inhibition of e.p.s.ps produced by 5-HT in the CA1 region of rat hippocampal slices. Br J Pharmacol. 124:93-100.

Radley, J.J., and B.L. Jacobs. 2002. 5-HT1A receptor antagonist administration decreases cell proliferation in the dentate gyrus. Brain Res. 955:264-267.

Ramachandrappa, S., and I.S. Farooqi. 2011. Genetic approaches to understanding human obesity. J Clin Invest. 121:2080-2086.

Ramboz, S., R. Oosting, D.A. Amara, H.F. Kung, P. Blier, M. Mendelsohn, J.J. Mann, D. Brunner, and R. Hen. 1998. Serotonin receptor 1A knockout: an animal model of anxiety-related disorder. Proc Natl Acad Sci U S A. 95:14476-14481.

Rapport, M.M., A.A. Green, and I.H. Page. 1948. Crystalline Serotonin. Science. 108:329-330.

Rayasam, G.V., V.K. Tulasi, R. Sodhi, J.A. Davis, and A. Ray. 2009. Glycogen synthase kinase 3: more than a namesake. Br J Pharmacol. 156:885-898.

Raymond, J.R., Y.V. Mukhin, A. Gelasco, J. Turner, G. Collinsworth, T.W. Gettys, J.S. Grewal, and M.N. Garnovskaya. 2001. Multiplicity of mechanisms of serotonin receptor signal transduction. Pharmacol Ther. 92:179-212.

Recio, J., P. Pevet, and M. Masson-Pevet. 1996. Serotonergic modulation of photically induced increase in melatonin receptor density and Fos immunoreactivity in the suprachiasmatic nuclei of the rat. J Neuroendocrinol. 8:839-845.

Reid, G., and M. Rand. 1952. Pharmacological actions of synthetic 5-hydroxytryptamine (serotonin, thrombocytin). Nature. 169:801-802.

Remington, G. 2008. Alterations of dopamine and serotonin transmission in schizophrenia. Prog Brain Res. 172:117-140.

Ren, H., I.J. Orozco, Y. Su, S. Suyama, R. Gutierrez-Juarez, T.L. Horvath, S.L. Wardlaw, L. Plum, O. Arancio, and D. Accili. 2012. FoxO1 target Gpr17 activates AgRP neurons to regulate food intake. Cell. 149:1314-1326.

Renehan, A.G., J. Frystyk, and A. Flyvbjerg. 2006. Obesity and cancer risk: the role of the insulin-IGF axis. Trends Endocrinol Metab. 17:328-336.

Renner, U., A. Zeug, A. Woehler, M. Niebert, A. Dityatev, G. Dityateva, N. Gorinski, D. Guseva, D. Abdel-Galil, M. Frohlich, F. Doring, E. Wischmeyer, D.W. Richter, E. Neher, and E.G. Ponimaskin. 2012. Heterodimerization of serotonin receptors 5-HT1A and 5-HT7 differentially regulates receptor signalling and trafficking. J Cell Sci. 125:2486-2499.

Reznik Off, L. 1960. Imipramine therapy of depressive syndromes. Am J Psychiatry. 116:1110-1111.

Riad, M., S. Garcia, K.C. Watkins, N. Jodoin, E. Doucet, X. Langlois, S. el Mestikawy, M. Hamon, and L. Descarries. 2000. Somatodendritic localization of 5-HT1A and preterminal axonal localization of 5-HT1B serotonin receptors in adult rat brain. J Comp Neurol. 417:181-194.

Roach, P.J. 1991. Multisite and hierarchal protein phosphorylation. J Biol Chem. 266:14139-14142.

Roepke, T.A., A.W. Smith, O.K. Ronnekleiv, and M.J. Kelly. 2012. Serotonin 5-HT2C receptor-mediated inhibition of the M-current in hypothalamic POMC neurons. Am J Physiol Endocrinol Metab. 302:E1399-1406.

Rohrschneider, L.R., J.F. Fuller, I. Wolf, Y. Liu, and D.M. Lucas. 2000. Structure, function, and biology of SHIP proteins. Genes Dev. 14:505-520.

Ropelle, E.R., J.R. Pauli, P. Prada, D.E. Cintra, G.Z. Rocha, J.C. Moraes, M.J. Frederico, G. da Luz, R.A. Pinho, J.B. Carvalheira, L.A. Velloso, M.A. Saad, and C.T. De Souza. 2009. Inhibition of hypothalamic Foxo1 expression reduced food intake in diet-induced obesity rats. J Physiol. 587:2341-2351.

Rosenzweig-Lipson, S., A. Sabb, G. Stack, P. Mitchell, I. Lucki, J.E. Malberg, S. Grauer, J. Brennan, J.F. Cryan, S.J. Sukoff Rizzo, J. Dunlop, J.E. Barrett, and K.L. Marquis. 2007. Antidepressant-like effects of the novel, selective, 5-HT2C receptor agonist WAY-163909 in rodents. Psychopharmacology (Berl). 192:159-170.

Roseweir, A.K., A.A. Katz, and R.P. Millar. 2012. Kisspeptin-10 inhibits cell migration in vitro via a receptor-GSK3 beta-FAK feedback loop in HTR8SVneo cells. Placenta. 33:408-415.

Roskoski, R., Jr. 2004. Src protein-tyrosine kinase structure and regulation. Biochem Biophys Res Commun. 324:1155-1164.

Rossi, J., N. Balthasar, D. Olson, M. Scott, E. Berglund, C.E. Lee, M.J. Choi, D. Lauzon, B.B. Lowell, and J.K. Elmquist. 2011. Melanocortin-4 receptors expressed by cholinergic neurons regulate energy balance and glucose homeostasis. Cell Metab. 13:195-204.

Rossi, M., M.S. Kim, D.G. Morgan, C.J. Small, C.M. Edwards, D. Sunter, S. Abusnana, A.P. Goldstone, S.H. Russell, S.A. Stanley, D.M. Smith, K. Yagaloff, M.A. Ghatei, and S.R. Bloom. 1998. A C-terminal fragment of Agouti-related protein increases feeding and antagonizes the effect of alpha-melanocyte stimulating hormone in vivo. Endocrinology. 139:4428-4431.

Roth, J., S. Qureshi, I. Whitford, M. Vranic, C.R. Kahn, I.G. Fantus, and J.H. Dirks. 2012. Insulin's discovery: new insights on its ninetieth birthday. Diabetes Metab Res Rev. 28:293-304.

Rother, E., B.F. Belgardt, E. Tsaousidou, B. Hampel, A. Waisman, M.G. Myers, Jr., and J.C. Bruning. 2012. Acute selective ablation of rat insulin promoter-expressing (RIPHER) neurons defines their orexigenic nature. Proc Natl Acad Sci U S A. 109:18132-18137.

Rothwell, N.J., and M.J. Stock. 1987. Effect of diet and fenfluramine on thermogenesis in the rat: possible involvement of serotonergic mechanisms. Int J Obes. 11:319-324.

Rousso-Noori, L., H. Knobler, E. Levy-Apter, Y. Kuperman, A. Neufeld-Cohen, Y. Keshet, V.R. Akepati, R.A. Klinghoffer, A. Chen, and A. Elson. 2011. Protein tyrosine phosphatase epsilon affects body weight by downregulating leptin signaling in a phosphorylation-dependent manner. Cell Metab. 13:562-572.

Rowland, N.E. 1986. Effect of continuous infusions of dexfenfluramine on food intake, body weight and brain amines in rats. Life Sci. 39:2581-2586.

Rowland, N.E., and J. Carlton. 1986. Neurobiology of an anorectic drug: fenfluramine. Prog Neurobiol. 27:13-62.

Rozakis-Adcock, M., J. McGlade, G. Mbamalu, G. Pelicci, R. Daly, W. Li, A. Batzer, S. Thomas, J. Brugge, P.G. Pelicci, and et al. 1992. Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. Nature. 360:689-692.

Ruat, M., E. Traiffort, R. Leurs, J. Tardivel-Lacombe, J. Diaz, J.M. Arrang, and J.C. Schwartz. 1993. Molecular cloning, characterization, and localization of a high-affinity serotonin receptor (5-HT7) activating cAMP formation. Proc Natl Acad Sci U S A. 90:8547-8551.

Rui, L., M. Yuan, D. Frantz, S. Shoelson, and M.F. White. 2002. SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. J Biol Chem. 277:42394-42398.

Ryan, D.H. 2004. Clinical use of sibutramine. Drugs Today (Barc). 40:41-54.

Rylatt, D.B., A. Aitken, T. Bilham, G.D. Condon, N. Embi, and P. Cohen. 1980. Glycogen synthase from rabbit skeletal muscle. Amino acid sequence at the sites phosphorylated by glycogen synthase kinase-3, and extension of the N-terminal sequence containing the site phosphorylated by phosphorylase kinase. Eur J Biochem. 107:529-537.

Sakowski, S.A., T.J. Geddes, D.M. Thomas, E. Levi, J.S. Hatfield, and D.M. Kuhn. 2006. Differential tissue distribution of tryptophan hydroxylase isoforms 1 and 2 as revealed with monospecific antibodies. Brain Res. 1085:11-18.

Salmeen, A., J.N. Andersen, M.P. Myers, N.K. Tonks, and D. Barford. 2000. Molecular basis for the dephosphorylation of the activation segment of the insulin receptor by protein tyrosine phosphatase 1B. Mol Cell. 6:1401-1412.

Sanberg, C.D., F.L. Jones, V.H. Do, D. Dieguez, Jr., and B.E. Derrick. 2006. 5-HT1a receptor antagonists block perforant path-dentate LTP induced in novel, but not familiar, environments. Learn Mem. 13:52-62.

Sanden, N., T. Thorlin, F. Blomstrand, P.A. Persson, and E. Hansson. 2000. 5-Hydroxytryptamine2B receptors stimulate Ca2+ increases in cultured astrocytes from three different brain regions. Neurochem Int. 36:427-434.

Santarelli, L., M. Saxe, C. Gross, A. Surget, F. Battaglia, S. Dulawa, N. Weisstaub, J. Lee, R. Duman, O. Arancio, C. Belzung, and R. Hen. 2003. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science. 301:805-809.

Santos, C.I., and A.P. Costa-Pereira. 2011. Signal transducers and activators of transcription-from cytokine signalling to cancer biology. Biochim Biophys Acta. 1816:38-49.

Sarbassov, D.D., D.A. Guertin, S.M. Ali, and D.M. Sabatini. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science. 307:1098-1101.

Sargent, B.J., and A.J. Henderson. 2011. Targeting 5-HT receptors for the treatment of obesity. Curr Opin Pharmacol. 11:52-58.

Sasaki, T., and T. Kitamura. 2010. Roles of FoxO1 and Sirt1 in the central regulation of food intake. Endocr J. 57:939-946.

Savitz, J., I. Lucki, and W.C. Drevets. 2009. 5-HT(1A) receptor function in major depressive disorder. Prog Neurobiol. 88:17-31.

Schmid, C.L., and L.M. Bohn. 2010. Serotonin, but not N-methyltryptamines, activates the serotonin 2A receptor via a ss-arrestin2/Src/Akt signaling complex in vivo. J Neurosci. 30:13513-13524.

Schmitt, A., R. Mossner, A. Gossmann, I.G. Fischer, V. Gorboulev, D.L. Murphy, H. Koepsell, and K.P. Lesch. 2003. Organic cation transporter capable of transporting serotonin is up-regulated in serotonin transporter-deficient mice. J Neurosci Res. 71:701-709.

Schulingkamp, R.J., T.C. Pagano, D. Hung, and R.B. Raffa. 2000. Insulin receptors and insulin action in the brain: review and clinical implications. Neurosci Biobehav Rev. 24:855-872.

Schultze, S.M., J. Jensen, B.A. Hemmings, O. Tschopp, and M. Niessen. 2011. Promiscuous affairs of PKB/AKT isoforms in metabolism. Arch Physiol Biochem. 117:70-77.

Scott, M.M., J.L. Lachey, S.M. Sternson, C.E. Lee, C.F. Elias, J.M. Friedman, and J.K. Elmquist. 2009. Leptin targets in the mouse brain. J Comp Neurol. 514:518-532.

Seino, S., M. Seino, S. Nishi, and G.I. Bell. 1989. Structure of the human insulin receptor gene and characterization of its promoter. Proc Natl Acad Sci U S A. 86:114-118.

Shanley, L.J., A.J. Irving, and J. Harvey. 2001. Leptin enhances NMDA receptor function and modulates hippocampal synaptic plasticity. J Neurosci. 21:RC186.

Shanley, L.J., A.J. Irving, M.G. Rae, M.L. Ashford, and J. Harvey. 2002a. Leptin inhibits rat hippocampal neurons via activation of large conductance calcium-activated K+ channels. Nat Neurosci. 5:299-300.

Shanley, L.J., D. O'Malley, A.J. Irving, M.L. Ashford, and J. Harvey. 2002b. Leptin inhibits epileptiform-like activity in rat hippocampal neurones via PI 3-kinase-driven activation of BK channels. J Physiol. 545:933-944.

Sharfi, H., and H. Eldar-Finkelman. 2008. Sequential phosphorylation of insulin receptor substrate-2 by glycogen synthase kinase-3 and c-Jun NH2-terminal kinase plays a role in hepatic insulin signaling. Am J Physiol Endocrinol Metab. 294:E307-315.

Sharma, S., and S. Fulton. 2012. Diet-induced obesity promotes depressive-like behaviour that is associated with neural adaptations in brain reward circuitry. Int J Obes (Lond).

Shaw, J.E., R.A. Sicree, and P.Z. Zimmet. 2010. Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pract. 87:4-14.

Sheline, Y.I., M.A. Mintun, D.M. Barch, C. Wilkins, A.Z. Snyder, and S.M. Moerlein. 2004. Decreased hippocampal 5-HT(2A) receptor binding in older depressed patients using [18F]altanserin positron emission tomography. Neuropsychopharmacology. 29:2235-2241.

Shibata, S., Y. Kagami-Ishi, K. Tominaga, K. Kodama, S. Ueki, and S. Watanabe. 1992. Ischemia-induced impairment of 2-deoxyglucose uptake and CA1 field potentials in rat hippocampal slices: protection by 5-HT1A receptor agonists and 5-HT2 receptor antagonists. Eur J Pharmacol. 229:21-29.

Shoelson, S.E., M. Sivaraja, K.P. Williams, P. Hu, J. Schlessinger, and M.A. Weiss. 1993. Specific phosphopeptide binding regulates a conformational change in the PI 3-kinase SH2 domain associated with enzyme activation. EMBO J. 12:795-802.

Siddle, K. 2012. Molecular basis of signaling specificity of insulin and IGF receptors: neglected corners and recent advances. Front Endocrinol (Lausanne). 3:34.

Skolnik, E.Y., C.H. Lee, A. Batzer, L.M. Vicentini, M. Zhou, R. Daly, M.J. Myers, Jr., J.M. Backer, A. Ullrich, M.F. White, and et al. 1993. The SH2/SH3 domain-containing protein GRB2 interacts with tyrosine-phosphorylated IRS1 and Shc: implications for insulin control of ras signalling. EMBO J. 12:1929-1936.

Sleeman, M.W., K.E. Wortley, K.M. Lai, L.C. Gowen, J. Kintner, W.O. Kline, K. Garcia, T.N. Stitt, G.D. Yancopoulos, S.J. Wiegand, and D.J. Glass. 2005. Absence of the lipid phosphatase SHIP2 confers resistance to dietary obesity. Nat Med. 11:199-205.

So, L., and D.A. Fruman. 2012. PI3K signalling in B- and T-lymphocytes: new developments and therapeutic advances. Biochem J. 442:465-481.

Soeda, Y., H. Tsuneki, H. Muranaka, N. Mori, S. Hosoh, Y. Ichihara, S. Kagawa, X. Wang, N. Toyooka, Y. Takamura, T. Uwano, H. Nishijo, T. Wada, and T. Sasaoka. 2010. The inositol phosphatase SHIP2 negatively regulates insulin/IGF-I actions implicated in neuroprotection and memory function in mouse brain. Mol Endocrinol. 24:1965-1977.

Sohn, J.W., Y. Xu, J.E. Jones, K. Wickman, K.W. Williams, and J.K. Elmquist. 2011. Serotonin 2C receptor activates a distinct population of arcuate pro-opiomelanocortin neurons via TRPC channels. Neuron. 71:488-497.

Song, M.S., L. Salmena, and P.P. Pandolfi. 2012. The functions and regulation of the PTEN tumour suppressor. Nat Rev Mol Cell Biol. 13:283-296.

Songyang, Z., S.E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W.G. Haser, F. King, T. Roberts, S. Ratnofsky, R.J. Lechleider, and et al. 1993. SH2 domains recognize specific phosphopeptide sequences. Cell. 72:767-778.

Soos, M.A., C.E. Field, and K. Siddle. 1993. Purified hybrid insulin/insulin-like growth factor-I receptors bind insulin-like growth factor-I, but not insulin, with high affinity. Biochem J. 290 (Pt 2):419-426.

Sotelo, C., B. Cholley, S. El Mestikawy, H. Gozlan, and M. Hamon. 1990. Direct Immunohistochemical Evidence of the Existence of 5-HT1A Autoreceptors on Serotoninergic Neurons in the Midbrain Raphe Nuclei. Eur J Neurosci. 2:1144-1154.

Sprouse, J.S., and G.K. Aghajanian. 1987. Electrophysiological responses of serotoninergic dorsal raphe neurons to 5-HT1A and 5-HT1B agonists. Synapse. 1:3-9.

Srinivasan, M., and N. Begum. 1994. Regulation of protein phosphatase 1 and 2A activities by insulin during myogenesis in rat skeletal muscle cells in culture. J Biol Chem. 269:12514-12520.

Staal, S.P. 1987. Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. Proc Natl Acad Sci U S A. 84:5034-5037.

Staal, S.P., J.W. Hartley, and W.P. Rowe. 1977. Isolation of transforming murine leukemia viruses from mice with a high incidence of spontaneous lymphoma. Proc Natl Acad Sci U S A. 74:3065-3067.

Stack, J.H., P.K. Herman, P.V. Schu, and S.D. Emr. 1993. A membrane-associated complex containing the Vps15 protein kinase and the Vps34 PI 3-kinase is essential for protein sorting to the yeast lysosome-like vacuole. EMBO J. 12:2195-2204.

Stahelin, R.V., D. Karathanassis, K.S. Bruzik, M.D. Waterfield, J. Bravo, R.L. Williams, and W. Cho. 2006. Structural and membrane binding analysis of the Phox homology domain of phosphoinositide 3-kinase-C2alpha. J Biol Chem. 281:39396-39406.

Starr, R., and D.J. Hilton. 1998. SOCS: suppressors of cytokine signalling. Int J Biochem Cell Biol. 30:1081-1085.

Steinbusch, H.W. 1981. Distribution of serotonin-immunoreactivity in the central nervous system of the rat-cell bodies and terminals. Neuroscience. 6:557-618.

Steiner, D.F., D. Cunningham, L. Spigelman, and B. Aten. 1967. Insulin biosynthesis: evidence for a precursor. Science. 157:697-700.

Stephens, L.R., A. Eguinoa, H. Erdjument-Bromage, M. Lui, F. Cooke, J. Coadwell, A.S. Smrcka, M. Thelen, K. Cadwallader, P. Tempst, and P.T. Hawkins. 1997. The G beta gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. Cell. 89:105-114.

Storlien, L.H., A.W. Thorburn, G.A. Smythe, A.B. Jenkins, D.J. Chisholm, and E.W. Kraegen. 1989. Effect of d-fenfluramine on basal glucose turnover and fat-feeding-induced insulin resistance in rats. Diabetes. 38:499-503.

Stoy, J., E.L. Edghill, S.E. Flanagan, H. Ye, V.P. Paz, A. Pluzhnikov, J.E. Below, M.G. Hayes, N.J. Cox, G.M. Lipkind, R.B. Lipton, S.A. Greeley, A.M. Patch, S. Ellard, D.F. Steiner, A.T. Hattersley, L.H. Philipson, and G.I. Bell. 2007. Insulin gene mutations as a cause of permanent neonatal diabetes. Proc Natl Acad Sci U S A. 104:15040-15044.

Stringhini, S., A.G. Tabak, T.N. Akbaraly, S. Sabia, M.J. Shipley, M.G. Marmot, E.J. Brunner, G.D. Batty, P. Bovet, and M. Kivimaki. 2012. Contribution of modifiable risk factors to social inequalities in type 2 diabetes: prospective Whitehall II cohort study. BMJ. 345:e5452.

Strubbe, J.H., and C.G. Mein. 1977. Increased feeding in response to bilateral injection of insulin antibodies in the VMH. Physiol Behav. 19:309-313.

Stumvoll, M., B.J. Goldstein, and T.W. van Haeften. 2005. Type 2 diabetes: principles of pathogenesis and therapy. Lancet. 365:1333-1346.

Sullivan, G.M., R.T. Ogden, M.A. Oquendo, J.S. Kumar, N. Simpson, Y.Y. Huang, J.J. Mann, and R.V. Parsey. 2009. Positron emission tomography quantification of serotonin-1A receptor binding in medication-free bipolar depression. Biol Psychiatry. 66:223-230.

Summers, S.A., A.W. Kao, A.D. Kohn, G.S. Backus, R.A. Roth, J.E. Pessin, and M.J. Birnbaum. 1999. The role of glycogen synthase kinase 3beta in insulin-stimulated glucose metabolism. J Biol Chem. 274:17934-17940.

Sun, X., H. Yao, R.M. Douglas, X.Q. Gu, J. Wang, and G.G. Haddad. 2010. Insulin/PI3K signaling protects dentate neurons from oxygen-glucose deprivation in organotypic slice cultures. J Neurochem. 112:377-388.

Sung, K.C., W.S. Jeong, S.H. Wild, and C.D. Byrne. 2012. Combined influence of insulin resistance, overweight/obesity, and fatty liver as risk factors for type 2 diabetes. Diabetes Care. 35:717-722.

Sutherland, C., I.A. Leighton, and P. Cohen. 1993. Inactivation of glycogen synthase kinase-3 beta by phosphorylation: new kinase connections in insulin and growth-factor signalling. Biochem J. 296 (Pt 1):15-19.

Suwa, A., T. Kurama, and T. Shimokawa. 2010. SHIP2 and its involvement in various diseases. Expert Opin Ther Targets. 14:727-737.

Sweet, L.J., B.D. Morrison, and J.E. Pessin. 1987. Isolation of functional alpha beta heterodimers from the purified human placental alpha 2 beta 2 heterotetrameric insulin receptor complex. A structural basis for insulin binding heterogeneity. J Biol Chem. 262:6939-6942.

Tam, J., R. Cinar, J. Liu, G. Godlewski, D. Wesley, T. Jourdan, G. Szanda, B. Mukhopadhyay, L. Chedester, J.S. Liow, R.B. Innis, K. Cheng, K.C. Rice, J.R. Deschamps, R.J. Chorvat, J.F. McElroy, and G. Kunos. 2012. Peripheral cannabinoid-1 receptor inverse agonism reduces obesity by reversing leptin resistance. Cell Metab. 16:167-179.

Taniguchi, C.M., B. Emanuelli, and C.R. Kahn. 2006. Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol. 7:85-96.

Tanji, C., H. Yamamoto, N. Yorioka, N. Kohno, K. Kikuchi, and A. Kikuchi. 2002. A-kinase anchoring protein AKAP220 binds to glycogen synthase kinase-3beta (GSK-3beta) and mediates protein kinase A-dependent inhibition of GSK-3beta. J Biol Chem. 277:36955-36961.

Tartaglia, L.A., M. Dembski, X. Weng, N. Deng, J. Culpepper, R. Devos, G.J. Richards, L.A. Campfield, F.T. Clark, J. Deeds, C. Muir, S. Sanker, A. Moriarty, K.J. Moore, J.S. Smutko, G.G. Mays, E.A. Wool, C.A. Monroe, and R.I. Tepper. 1995. Identification and expression cloning of a leptin receptor, OB-R. Cell. 83:1263-1271.

Tatemoto, K. 1982. Neuropeptide Y: complete amino acid sequence of the brain peptide. Proc Natl Acad Sci U S A. 79:5485-5489.

Tavare, J.M., B. Clack, and L. Ellis. 1991. Two-dimensional phosphopeptide analysis of the autophosphorylation cascade of a soluble insulin receptor tyrosine kinase. The tyrosines phosphorylated are typical of those observed following phosphorylation of the heterotetrameric insulin receptor in intact cells. J Biol Chem. 266:1390-1395.

Tecott, L.H., A.V. Maricq, and D. Julius. 1993. Nervous system distribution of the serotonin 5-HT3 receptor mRNA. Proc Natl Acad Sci U S A. 90:1430-1434.

Tecott, L.H., L.M. Sun, S.F. Akana, A.M. Strack, D.H. Lowenstein, M.F. Dallman, and D. Julius. 1995. Eating disorder and epilepsy in mice lacking 5-HT2c serotonin receptors. Nature. 374:542-546.

ten Hoeve, J., M. de Jesus Ibarra-Sanchez, Y. Fu, W. Zhu, M. Tremblay, M. David, and K. Shuai. 2002. Identification of a nuclear Stat1 protein tyrosine phosphatase. Mol Cell Biol. 22:5662-5668.

Terruzzi, I., S. Allibardi, P. Bendinelli, P. Maroni, R. Piccoletti, F. Vesco, M. Samaja, and L. Luzi. 2002. Amino acid- and lipid-induced insulin resistance in rat heart: molecular mechanisms. Mol Cell Endocrinol. 190:135-145.

Thornton, T.M., G. Pedraza-Alva, B. Deng, C.D. Wood, A. Aronshtam, J.L. Clements, G. Sabio, R.J. Davis, D.E. Matthews, B. Doble, and M. Rincon. 2008. Phosphorylation by p38 MAPK as an alternative pathway for GSK3beta inactivation. Science. 320:667-670.

Tsuboi, N., T. Utsunomiya, R.L. Roberts, H. Ito, K. Takahashi, M. Noda, and T. Takahashi. 2008. The tyrosine phosphatase CD148 interacts with the p85 regulatory subunit of phosphoinositide 3-kinase. Biochem J. 413:193-200.

Twomey, C., and J.V. McCarthy. 2006. Presenilin-1 is an unprimed glycogen synthase kinase-3beta substrate. FEBS Lett. 580:4015-4020.

Tzivion, G., M. Dobson, and G. Ramakrishnan. 2011. FoxO transcription factors; Regulation by AKT and 14-3-3 proteins. Biochim Biophys Acta. 1813:1938-1945.

Ueki, K., T. Kondo, and C.R. Kahn. 2004. Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms. Mol Cell Biol. 24:5434-5446.

Ugi, S., T. Imamura, H. Maegawa, K. Egawa, T. Yoshizaki, K. Shi, T. Obata, Y. Ebina, A. Kashiwagi, and J.M. Olefsky. 2004. Protein phosphatase 2A negatively regulates insulin's metabolic signaling pathway by inhibiting Akt (protein kinase B) activity in 3T3-L1 adipocytes. Mol Cell Biol. 24:8778-8789.

Ullrich, A., J.R. Bell, E.Y. Chen, R. Herrera, L.M. Petruzzelli, T.J. Dull, A. Gray, L. Coussens, Y.C. Liao, M. Tsubokawa, and et al. 1985. Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. Nature. 313:756-761.

Ullrich, A., and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. Cell. 61:203-212.

Vadas, O., J.E. Burke, X. Zhang, A. Berndt, and R.L. Williams. 2011. Structural basis for activation and inhibition of class I phosphoinositide 3-kinases. Sci Signal. 4:re2.

van de Wall, E., R. Leshan, A.W. Xu, N. Balthasar, R. Coppari, S.M. Liu, Y.H. Jo, R.G. MacKenzie, D.B. Allison, N.J. Dun, J. Elmquist, B.B. Lowell, G.S. Barsh, C. de Luca, M.G. Myers, Jr., G.J. Schwartz, and S.C. Chua, Jr. 2008. Collective and individual functions of leptin receptor modulated neurons controlling metabolism and ingestion. Endocrinology. 149:1773-1785.

van der Geer, P., T. Hunter, and R.A. Lindberg. 1994. Receptor protein-tyrosine kinases and their signal transduction pathways. Annu Rev Cell Biol. 10:251-337.

van der Heide, L.P., G.M. Ramakers, and M.P. Smidt. 2006. Insulin signaling in the central nervous system: learning to survive. Prog Neurobiol. 79:205-221.

van Hooft, J.A., and J.L. Yakel. 2003. 5-HT3 receptors in the CNS: 3B or not 3B? Trends Pharmacol Sci. 24:157-160.

van Weeren, P.C., K.M. de Bruyn, A.M. de Vries-Smits, J. van Lint, and B.M. Burgering. 1998. Essential role for protein kinase B (PKB) in insulin-induced glycogen synthase kinase 3 inactivation. Characterization of dominant-negative mutant of PKB. J Biol Chem. 273:13150-13156.

Vanhaesebroeck, B., J. Guillermet-Guibert, M. Graupera, and B. Bilanges. 2010. The emerging mechanisms of isoform-specific PI3K signalling. Nat Rev Mol Cell Biol. 11:329-341.

Vanhaesebroeck, B., S.J. Leevers, G. Panayotou, and M.D. Waterfield. 1997. Phosphoinositide 3-kinases: a conserved family of signal transducers. Trends Biochem Sci. 22:267-272.

Vanhaesebroeck, B., L. Stephens, and P. Hawkins. 2012. PI3K signalling: the path to discovery and understanding. Nat Rev Mol Cell Biol. 13:195-203.

Verge, D., G. Daval, M. Marcinkiewicz, A. Patey, S. el Mestikawy, H. Gozlan, and M. Hamon. 1986. Quantitative autoradiography of multiple 5-HT1 receptor subtypes in the brain of control or 5,7-dihydroxytryptamine-treated rats. J Neurosci. 6:3474-3482.

Vickers, S.P., K.R. Benwell, R.H. Porter, M.J. Bickerdike, G.A. Kennett, and C.T. Dourish. 2000. Comparative effects of continuous infusion of mCPP, Ro 60-0175 and d-fenfluramine on food intake, water intake, body weight and locomotor activity in rats. Br J Pharmacol. 130:1305-1314.

Vickers, S.P., and C.T. Dourish. 2004. Serotonin receptor ligands and the treatment of obesity. Curr Opin Investig Drugs. 5:377-388.

Vilaro, M.T., R. Cortes, C. Gerald, T.A. Branchek, J.M. Palacios, and G. Mengod. 1996. Localization of 5-HT4 receptor mRNA in rat brain by in situ hybridization histochemistry. Brain Res Mol Brain Res. 43:356-360.

Vilaro, M.T., R. Cortes, and G. Mengod. 2005. Serotonin 5-HT4 receptors and their mRNAs in rat and guinea pig brain: distribution and effects of neurotoxic lesions. J Comp Neurol. 484:418-439.

Volinia, S., R. Dhand, B. Vanhaesebroeck, L.K. MacDougall, R. Stein, M.J. Zvelebil, J. Domin, C. Panaretou, and M.D. Waterfield. 1995. A human phosphatidylinositol 3-kinase complex related to the yeast Vps34p-Vps15p protein sorting system. EMBO J. 14:3339-3348.

Vucenik, I., and J.P. Stains. 2012. Obesity and cancer risk: evidence, mechanisms, and recommendations. Ann N Y Acad Sci. 1271:37-43.

Wan, Q., Z.G. Xiong, H.Y. Man, C.A. Ackerley, J. Braunton, W.Y. Lu, L.E. Becker, J.F. MacDonald, and Y.T. Wang. 1997. Recruitment of functional GABA(A) receptors to postsynaptic domains by insulin. Nature. 388:686-690.

Wang, M.Y., Y. Lee, and R.H. Unger. 1999. Novel form of lipolysis induced by leptin. J Biol Chem. 274:17541-17544.

Wang, M.Y., L. Orci, M. Ravazzola, and R.H. Unger. 2005. Fat storage in adipocytes requires inactivation of leptin's paracrine activity: implications for treatment of human obesity. Proc Natl Acad Sci U S A. 102:18011-18016.

Wauman, J., and J. Tavernier. 2011. Leptin receptor signaling: pathways to leptin resistance. Front Biosci. 16:2771-2793.

Wearing, S.C., E.M. Hennig, N.M. Byrne, J.R. Steele, and A.P. Hills. 2006. Musculoskeletal disorders associated with obesity: a biomechanical perspective. Obes Rev. 7:239-250.

Wieland, S., and I. Lucki. 1990. Antidepressant-like activity of 5-HT1A agonists measured with the forced swim test. Psychopharmacology (Berl). 101:497-504.

Williams, K.W., L.O. Margatho, C.E. Lee, M. Choi, S. Lee, M.M. Scott, C.F. Elias, and J.K. Elmquist. 2010. Segregation of acute leptin and insulin effects in distinct populations of arcuate proopiomelanocortin neurons. J Neurosci. 30:2472-2479.

Williams, K.W., M.M. Scott, and J.K. Elmquist. 2011. Modulation of the central melanocortin system by leptin, insulin, and serotonin: co-ordinated actions in a dispersed neuronal network. Eur J Pharmacol. 660:2-12.

Willoughby, J.O., and W.W. Blessing. 1987. Origin of serotonin innervation of the arcuate and ventromedial hypothalamic region. Brain Res. 418:170-173.

Wilson, L.S., G.S. Baillie, L.M. Pritchard, B. Umana, A. Terrin, M. Zaccolo, M.D. Houslay, and D.H. Maurice. 2011. A phosphodiesterase 3B-based signaling complex integrates exchange protein activated by cAMP 1 and phosphatidylinositol 3-kinase signals in human arterial endothelial cells. J Biol Chem. 286:16285-16296.

Wisniewski, D., A. Strife, S. Swendeman, H. Erdjument-Bromage, S. Geromanos, W.M. Kavanaugh, P. Tempst, and B. Clarkson. 1999. A novel SH2-containing phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase (SHIP2) is constitutively tyrosine phosphorylated and associated with src homologous and collagen gene (SHC) in chronic myelogenous leukemia progenitor cells. Blood. 93:2707-2720.

Wolf, G., T. Trub, E. Ottinger, L. Groninga, A. Lynch, M.F. White, M. Miyazaki, J. Lee, and S.E. Shoelson. 1995. PTB domains of IRS-1 and Shc have distinct but overlapping binding specificities. J Biol Chem. 270:27407-27410.

Woodgett, J.R. 1990. Molecular cloning and expression of glycogen synthase kinase-3/factor A. EMBO J. 9:2431-2438.

Wouters, M.M., J.L. Roeder, V.S. Tharayil, J.E. Stanich, P.R. Strege, S. Lei, M.R. Bardsley, T. Ordog, S.J. Gibbons, and G. Farrugia. 2009. Protein kinase C{gamma} mediates regulation of proliferation by the serotonin 5-hydroxytryptamine receptor 2B. J Biol Chem. 284:21177-21184.

Wu, H., S.C. Shekar, R.J. Flinn, M. El-Sibai, B.S. Jaiswal, K.I. Sen, V. Janakiraman, S. Seshagiri, G.J. Gerfen, M.E. Girvin, and J.M. Backer. 2009. Regulation of Class IA PI 3-kinases: C2 domain-iSH2 domain contacts inhibit p85/p110alpha and are disrupted in oncogenic p85 mutants. Proc Natl Acad Sci U S A. 106:20258-20263.

Xi, G., X. Shen, and D.R. Clemmons. 2010. p66shc inhibits insulin-like growth factor-I signaling via direct binding to Src through its polyproline and Src homology 2 domains, resulting in impairment of Src kinase activation. J Biol Chem. 285:6937-6951.

Xu, A.W., C.B. Kaelin, K. Takeda, S. Akira, M.W. Schwartz, and G.S. Barsh. 2005. PI3K integrates the action of insulin and leptin on hypothalamic neurons. J Clin Invest. 115:951-958.

Xu, Y., E.D. Berglund, J.W. Sohn, W.L. Holland, J.C. Chuang, M. Fukuda, J. Rossi, K.W. Williams, J.E. Jones, J.M. Zigman, B.B. Lowell, P.E. Scherer, and J.K. Elmquist. 2010a. 5-HT2CRs expressed by pro-opiomelanocortin neurons regulate insulin sensitivity in liver. Nat Neurosci. 13:1457-1459.

Xu, Y., J.E. Jones, D. Kohno, K.W. Williams, C.E. Lee, M.J. Choi, J.G. Anderson, L.K. Heisler, J.M. Zigman, B.B. Lowell, and J.K. Elmquist. 2008. 5-HT2CRs expressed by pro-opiomelanocortin neurons regulate energy homeostasis. Neuron. 60:582-589.

Xu, Y., J.E. Jones, D.A. Lauzon, J.G. Anderson, N. Balthasar, L.K. Heisler, A.R. Zinn, B.B. Lowell, and J.K. Elmquist. 2010b. A serotonin and melanocortin circuit mediates D-fenfluramine anorexia. J Neurosci. 30:14630-14634.

Yadav, V.K., F. Oury, N. Suda, Z.W. Liu, X.B. Gao, C. Confavreux, K.C. Klemenhagen, K.F. Tanaka, J.A. Gingrich, X.E. Guo, L.H. Tecott, J.J. Mann, R. Hen, T.L. Horvath, and G. Karsenty. 2009. A serotonin-dependent mechanism explains the leptin regulation of bone mass, appetite, and energy expenditure. Cell. 138:976-989.

Yadav, V.K., F. Oury, K.F. Tanaka, T. Thomas, Y. Wang, S. Cremers, R. Hen, A. Krust, P. Chambon, and G. Karsenty. 2011. Leptin-dependent serotonin control of appetite: temporal specificity, transcriptional regulation, and therapeutic implications. J Exp Med. 208:41-52.

Yamada, N., G. Katsuura, Y. Ochi, K. Ebihara, T. Kusakabe, K. Hosoda, and K. Nakao. 2011. Impaired CNS leptin action is implicated in depression associated with obesity. Endocrinology. 152:2634-2643.

Yang, S.B., A.C. Tien, G. Boddupalli, A.W. Xu, Y.N. Jan, and L.Y. Jan. 2012. Rapamycin ameliorates age-dependent obesity associated with increased mTOR signaling in hypothalamic POMC neurons. Neuron. 75:425-436.

Yip, S.C., S. Saha, and J. Chernoff. 2010. PTP1B: a double agent in metabolism and oncogenesis. Trends Biochem Sci. 35:442-449.

Yu, J., Y. Zhang, J. McIlroy, T. Rordorf-Nikolic, G.A. Orr, and J.M. Backer. 1998. Regulation of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit. Mol Cell Biol. 18:1379-1387.

Zabolotny, J.M., K.K. Bence-Hanulec, A. Stricker-Krongrad, F. Haj, Y. Wang, Y. Minokoshi, Y.B. Kim, J.K. Elmquist, L.A. Tartaglia, B.B. Kahn, and B.G. Neel. 2002. PTP1B regulates leptin signal transduction in vivo. Dev Cell. 2:489-495.

Zahniser, N.R., M.B. Goens, P.J. Hanaway, and J.V. Vinych. 1984. Characterization and regulation of insulin receptors in rat brain. J Neurochem. 42:1354-1362.

Zhang, B., J.M. Tavare, L. Ellis, and R.A. Roth. 1991. The regulatory role of known tyrosine autophosphorylation sites of the insulin receptor kinase domain. An assessment by replacement with neutral and negatively charged amino acids. J Biol Chem. 266:990-996.

Zhang, E.E., E. Chapeau, K. Hagihara, and G.S. Feng. 2004. Neuronal Shp2 tyrosine phosphatase controls energy balance and metabolism. Proc Natl Acad Sci U S A. 101:16064-16069.

Zhang, F., M.B. Basinski, J.M. Beals, S.L. Briggs, L.M. Churgay, D.K. Clawson, R.D. DiMarchi, T.C. Furman, J.E. Hale, H.M. Hsiung, B.E. Schoner, D.P. Smith, X.Y. Zhang, J.P. Wery, and R.W. Schevitz. 1997. Crystal structure of the obese protein leptin-E100. Nature. 387:206-209.

Zhang, F., and J. Chen. 2008. Leptin protects hippocampal CA1 neurons against ischemic injury. J Neurochem. 107:578-587.

Zhang, X., R.R. Gainetdinov, J.M. Beaulieu, T.D. Sotnikova, L.H. Burch, R.B. Williams, D.A. Schwartz, K.R. Krishnan, and M.G. Caron. 2005. Loss-of-function mutation in tryptophan hydroxylase-2 identified in unipolar major depression. Neuron. 45:11-16.

Zhang, X., O. Vadas, O. Perisic, K.E. Anderson, J. Clark, P.T. Hawkins, L.R. Stephens, and R.L. Williams. 2011. Structure of lipid kinase p110beta/p85beta elucidates an unusual SH2-domain-mediated inhibitory mechanism. Mol Cell. 41:567-578.

Zhang, Y., R. Proenca, M. Maffei, M. Barone, L. Leopold, and J.M. Friedman. 1994. Positional cloning of the mouse obese gene and its human homologue. Nature. 372:425-432.

Zhao, L., K.W. Kim, Y. Ikeda, K.K. Anderson, L. Beck, S. Chase, S.A. Tobet, and K.L. Parker. 2008. Central nervous system-specific knockout of steroidogenic factor 1 results in increased anxiety-like behavior. Mol Endocrinol. 22:1403-1415.

Zhao, W., H. Chen, H. Xu, E. Moore, N. Meiri, M.J. Quon, and D.L. Alkon. 1999. Brain insulin receptors and spatial memory. Correlated changes in gene expression, tyrosine phosphorylation, and signaling molecules in the hippocampus of water maze trained rats. J Biol Chem. 274:34893-34902.

Zhao, W.Q., H. Chen, M.J. Quon, and D.L. Alkon. 2004. Insulin and the insulin receptor in experimental models of learning and memory. Eur J Pharmacol. 490:71-81.

Zhou, L., G.M. Sutton, J.J. Rochford, R.K. Semple, D.D. Lam, L.J. Oksanen, Z.D. Thornton-Jones, P.G. Clifton, C.Y. Yueh, M.L. Evans, R.J. McCrimmon, J.K. Elmquist, A.A. Butler, and L.K. Heisler. 2007. Serotonin 2C receptor agonists improve type 2 diabetes via melanocortin-4 receptor signaling pathways. Cell Metab. 6:398-405.

Résumé

L'insuline et la sérotonine (5-HT) sont deux acteurs majeurs du maintien de l'homéostasie énergétique, fonction placée sous le contrôle de l'hypothalamus. En ciblant cette région, l'insuline remplit de nombreuses fonctions métaboliques via l'activation de la voie PI3K/Akt. La 5-HT exercent des effets biologiques similaires mais les voies de signalisation impliquées dans ces processus étaient jusqu'alors mal connues. De plus, il avait été démontré que la 5-HT est capable d'activer la voie PI3K/Akt/GSK3β dans l'hippocampe, mécanisme sous-tendant potentiellement les effets antidépresseurs du neurotransmetteur.

Les principaux objectifs de cette thèse étaient d'étudier 1/ l'activation de la voie PI3K/Akt par la 5-HT dans l'hypothalamus de rats diabétiques (modèle Goto-Kakizaki) et chercher un potentiel dialogue avec l'insuline and 2/ les mécanismes sous-tendant l'induction de la dépression par une alimentation hyperlipidique, par l'analyse de la phosphorylation d'Akt et GSK3β sous l'action de l'insuline, de la leptine et de la 5-HT dans l'hippocampe de rat.

Ici on montre que 1/ la 5-HT stimule la voie PI3K/Akt dans l'hypothalamus et que la phosphorylation d'Akt induite par la 5-HT est atténuée dans des conditions d'insulino-résistance, suggérant l'existence d'un dialogue entre les voies de signalisation de l'insuline et de la 5-HT. Par ailleurs, nos résultats indiquent qu'une alimentation hyperlipidique induit un comportement dépressif réversible chez le rat, qui pourrait impliquer la voie PI3K/Akt/GSK3β dans les neurones subgranulaires du gyrus denté.

La mise en évidence d'un dialogue entre les voies de signalisation de la 5-HT, de la leptine et de l'insuline au niveau central enrichit nos connaissances sur le rôle de ces facteurs dans la régulation de l'homéostasie énergétique et de l'humeur, et propose un lien moléculaire entre diabète de type 2, obésité et dépression.

Abstract

Insulin and serotonin (5-HT) are two key players in the maintenance of energy homeostasis which is controlled by the hypothalamus. In this brain region, insulin mediates numerous metabolic effects via the activation of the PI3K/Akt signaling pathway. 5-HT exerts similar biological properties by acting in the hypothalamus but the signaling pathways accountable for these effects are still unclear. Moreover, it has been reported that 5-HT induces the activation of the PI3K/Akt pathway in the hippocampus and the inhibition of GSK3β, suggesting this action as a potential mechanism for the antidepressant effects of this neurotransmitter.

The main objectives of this thesis were to study 1/ the serotonin-induced activation of the PI3K/Akt in the hypothalamus of wild type and diabetic rats (Goto-Kakizaki model) and search a potential cross-talk with insulin and, 2/ the mechanisms underlying the high-fat diet induced depression by investigating the role of the phosphorylation of Akt and GSK3β by 5-HT, insulin and leptin in the hippocampus of rats.

Here, we show that 5-HT triggers the PI3K/Akt signaling pathway in the rat hypothalamus, and that this activation is attenuated in insulin-resistant conditions, suggesting a cross-talk between insulin and 5-HT. Moreover, we reported that high-fat diet feeding induces a reversible depressive-like behavior, which may involve the PI3K/Akt/GSK3β pathway in subgranular neurons of the dentate gyrus.

In conclusion, the activation of the PI3K/Akt pathway and its target GSK3β by 5-HT in the hypothalamus and in the dentate gyrus, respectively, can be impaired in insulin-/leptin-resistant states, which may underlie a link between metabolic diseases and depression.