The role of NPY in seizure control and weight gain when valproic acid is used by epilepsy patients

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Aims: That weight gain is one of the concerning side effects of valproic acid (VPA) is confirmed repeatedly in convincing studies. VPA is mainly used by epileptic patients and been available on the market for many years. VPA suppresses susceptibility to seizures. However, the underlying mechanism of it and why it induces weight gain remains unknown. Several hypotheses have been suggested to explain the observed effects of VPA. This thesis focuses on one of these hypotheses, namely the role of neuropeptide Y (NPY) in VPA-induced weight gain. We hypothesized that VPA induce weight gain by increasing the levels of NPY.

Methods: Initially, the role of NPY in seizure control is elaborated. Subsequently, the role of NPY in energy homeostasis is described. In the third chapter we attempt to make a link between VPA and NPY. Finally, other possible hypotheses to explain VPA-induced weight gain are discussed.

Results: NPY is a neuropeptide that mainly functions in the brain. NPY is involved in seizure control and energy homeostasis. In seizure control NPY reduces susceptibility to seizures. In energy homeostasis it increases appetite and thereby food intake. NPY levels are enhanced after starting with VPA. However, measurements were done several months after starting the VPA-therapy. In addition, the levels of other neuronal peptides and hormones were changed.

Conclusion: Because NPY plays a role in both seizure and weight control, NPY is a possible candidate for the link between VPA and weight gain. However, it is difficult to establish if VPA is affecting NPY directly or indirectly. Changes in NPY levels could also be a secondary consequence of VPA therapy and more hypotheses could be mentioned to explain VPA-induced weight gain. Therefore, more research is needed to investigate these hypotheses.

Keywords: valproic acid, neuropeptide Y, weight gain, energy metabolism.

Abbreviations

VPA	Valproic Acid
NPY	Neuropeptide Y
KO	Knock-out
GABA	Gamma-aminobutyric acid
KA	Kainic acid
rAAV	recombinant adeno-associated virus
TLE	Temporal lobe epilepsy
FS	Febrile seizures
MAPK	Mitogen-activated protein kinase
FC	Febrile convulsions
PTZ	Pentylenetetrazole
PV	Paravalbumin
PVN	Paraventricular nucleus
DMH	Dorsomedial hypothalamus
ARC	Arcuate nucleus
AgRP	Agoutirelated peptide
POMC	Pro-opiomelanocortin
CART	Cocaine and amphetamine-regulated transcript
LHA	Lateral hypothalamus
PFA	Perifornical area
CCK	Cholecystokinin
GLP-1	Glucogon-like peptide-1
PYY	Peptide YY
BDNF	Brain-derived neurotropic factor
TrkB	Tyrosine-related kinase B
Erk	Extracellular singal-regulated kinase
FFA	Free fatty acids
RSTN	Resistin
SOC-3	Suppressor of cytokine signaling-3
FIAF	Fasting-induced adipose factor
C/EBPα	CCAAT-enhancer-binding protein
AP	Allopregnanolone

1. Introduction

Epilepsy is a chronic neurological disorder that is characterized by seizures. Many people worldwide suffer from this disease. Fortunately for most patients their disease can be controlled with effective medicine. Valproic acid (VPA) is one of the most frequently-prescribed antiepileptic drugs and it is used to treat various types of epilepsy (Gerstner et al., 2008). In addition to its use for epilepsy, VPA is used in the treatment of various other diseases such as bipolar disorders (Smith et al., 2010) and migraine prophylaxes (Vikelis and Rapoport, 2010). Apart from the appropriate working of this medicine it may induce side effects (Gerstner et al., 2008). The most common side effects for mothers are an increased risk of neural tube defects and other congenital neonatal diseases in the offspring (Ornoy, 2009). In addition, thrombocytopenia is one of the side effects (Nasreddine and Beydoun, 2008). Furthermore, many patients suffer from weight gain after starting VPA therapy (Dinesen et al., 1984;Corman et al., 1997;Aydin et al., 2005;El-Khatib et al., 2007;Hamed et al., 2009;Martin et al., 2009;Cansu et al., 2011). This thesis will focus on the latter, unpleasant and dangerous side effect weight gain by patients using VPA.

Significant weight gain after starting VPA treatment is seen in both genders. However women are more prone to gain weight and suffer more from sociopsychological burden of being overweight than men (El-Khatib et al., 2007). Gaining weight is a troubling side effect, because overweight and obese patients face a higher risk of infertility (Van, 2011), development of diabetes (Das and Mukhopadhyay, 2011) or cardiovascular problems (Aprahamian and Sam, 2011). Several mechanisms have been suggested to be involved in VPA-induced weight gain (Figure 1) (Verrotti et al., 2011). This thesis will focus on the effect of VPA on the hypothalamus and especially on the role of neuropeptide Y (NPY) in seizure control and weight gain.

NPY, a 36-amino-acid neurotransmitter, is distributed and functions throughout the peripheral en central nervous system. NPY is assumed to be involved in many regulatory mechanisms such as the modulation of mood (Thorsell, 2010), bone

remodeling (Lee and Herzog, 2009) and vascular remodeling (Abe et al., 2007). Chapter 2 of this thesis will describe how NPY is involved in seizure control. In chapter 3 the role of NPY in energy homeostasis will be summarized. In seizure control, NPY suppresses susceptibility to seizures. In energy homeostasis, NPY increases appetite and thereby food intake. Possibly, NPY is the link between the appropriate working of VPA and its side effect weight gain. In this thesis the link between VPA and NPY will be elaborated.

The mechanism relating VPA and weight gain is probably multifactorial. Therefore, in the last chapter of this thesis other possible mechanism that could be involved in or interacting with VPA-induced weight gain will be described briefly. By discussing this questions and the questions described above we hope to find out the role of NPY in seizure control and weight gain when VPA is used to treat epileptic patients.

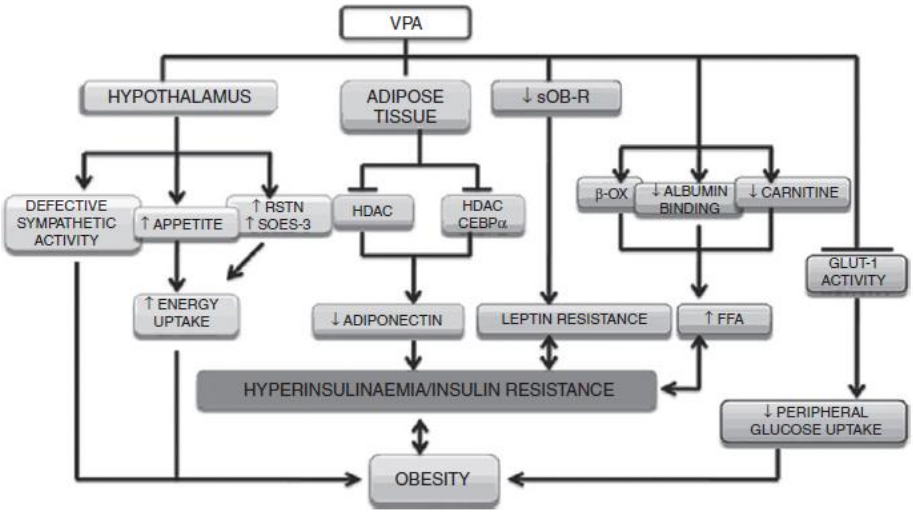

Figure 1: Mechanisms involved in VPA-induced weight gain. β-OX, beta-oxidation; C/EBPα, CCAAT/enhancer binding protein; FFA, free fatty acid; HDAC, histone deacetylase activity; RSTN, resistin; SOCS-3, cytokine signaling-3; VPA, valproic acid (Verrotti et al., 2011).

2. The role of NPY in seizure control in epileptic patients

NPY is a small neuropeptide and fibers containing this peptide are found in both the central en the peripheral nervous system (Chronwall and Zukowska, 2004). The peptide is acting throughout the whole nervous system. However, it acts predominantly and is mainly present in various regions in the brain. NPY mediates its effect through the activation of five different receptors (NPY receptors 1-5, usually denoted Y1, Y2 etc.). These G protein-coupled NPY receptors are, like the neuropeptide itself, distributed throughout the whole nervous system as well as in other tissues.

NPY affects many processes, for instance the modulation of mood, bone remodeling, food intake and cardiovascular processes. In addition, NPY is thought to play a role in seizure control in epileptic patients. The presence of NPY and its receptors is observed in regions known to be important in seizure control, which are mainly regions in the hippocampus. The role of NPY in seizure control has been examined repeatedly (e.g. (Erickson et al., 1996;Baraban et al., 1997;Richichi et al., 2004;Woldbye et al., 2005) and has been comprehensively reviewed (Colmers and El, 2003;Baraban, 2004). In this section most important evidence, to demonstrate that NPY is involved in seizure control, will be summarized and discussed.

2.1 Suppression of NPY increases susceptibility to seizures

The inhibitory effect of NPY on neuronal excitability and therefore on seizure control is predominantly studied in *in vitro* and *in vivo* experiments using rodents. A higher susceptibility to seizures is observed in NPY knock-out (KO) and mutant rodents that had no detectable levels of NPY (Erickson et al., 1996;Baraban et al., 1997;DePrato et al., 2000;Weinshenker et al., 2001). Susceptibility to seizures is mainly tested with drugs that induce seizures such as gamma-aminobutyric (GABA) antagonists and kainic acid (KA). For instance, NPY-deficient mice were more susceptible to mild seizures in response to GABA antagonists (Erickson et al., 1996). In addition, these NPY-deficient mice showed more severe seizures. Furthermore, giving KA to NPY-deficient mice led to the death of 93% of these mice (Baraban et al., 1997). NPY infusion, before the infusion of KA,

prevented death in NPY-deficient mice. An increase in spontaneous seizures after suppression of NPY was not observed.

2.2 NPY overexpression reduces susceptibility to seizures

By showing that NPY-deficient mice are more susceptible to seizures, it is interesting to examine the effect of overexpressing NPY in rodents. Transgenic rats that overexpress NPY were less susceptible to seizures compared to WT-rats (Vezzani et al., 2002). In addition, a protecting effect of NPY was observed in experiments using rodents that received high amounts of NPY by injections in brain areas that are important in seizure control (Woldbye et al., 1997;Mazarati and Wasterlain, 2002). For example, NPY administration into the lateral ventricle of rats and mice inhibited electroencephalographic seizures induced by KA (Woldbye et al., 1997).

Furthermore, an increase of NPY in specific brain parts was induced by local application of recombinant adeno-associated virus (rAAV) vectors (Richichi et al., 2004;Foti et al., 2007;Noe et al., 2009;Noe et al., 2010). rAAV vectors are viruses that do not cause diseases and can be used for gene transfer. By using these vectors DNA strands, containing transcripts for genes (e.g. the *NPY* gene), will be incorporated in host target cells for instance neuronal cells. Noe and colleagues induced overexpression of *NPY* in rat hippocampuses by using a vector (Noe et al., 2008;Noe et al., 2010). They observed a protective effect of NPY on seizure control. In addition, *NPY* gene therapy decreased (by 40%) chronic spontaneous seizures in a rat model of temporal lobe epilepsy (TLE) (Noe et al., 2008). This effect was correlated with NPY overexpression in the hippocampus.

2.3 NPY and NPY receptors are increased after seizures

From sections 2.1 and 2.2 it can be concluded that NPY suppresses susceptibility to seizures. In addition to this, NPY levels and the presence of NPY receptors increase after seizures, probably as a compensatory mechanism. NPY containing neurons are increased in epileptic animal models (Marksteiner and Sperk, 1988;Marksteiner et al., 1989;Sperk et al., 1992;Mikkelsen et al., 1994;Schwarzer et al., 1996;Dube, 2007;Cardoso et al.,

2010) particular in the granular and pyramidal cells (Xapelli et al., 2006). For example, in a rat model of febrile seizures (FS), the most common type of seizures in infants and young children, NPY expression was up-regulated in hippocampus after experimentally induced FS (Dube, 2007). This up-regulation was associated with an increased seizure threshold for additional recurrent FS.

2.4 The molecular mechanism of NPY-related seizure control

Susceptibility to seizures can be described as an imbalance among excitation and inhibition. Excitation is mediated by the neurotransmitter glutamate, whereas inhibition results mainly from the action of GABA (Colmers and El Bahn, 2003). NPY is inhibiting excitation by inhibiting glutamate release from Schaffer collateral/CA1 synapses and at mossy fibers/CA3 synapses (Colmers et al., 1985;Colmers et al., 1991;Bleakman et al., 1991;Klapstein and Colmers, 1992;Greber et al., 1994;Klapstein and Colmers, 1997;Xapelli et al., 2006). NPY is acting presynaptically and most likely directly at the terminal ends of neurons to inhibit CA^{2+} currents, which leads to the suppression of depolarization-induced glutamate release. This effect, also described as the inhibition of synaptic transmission, is mediated by different NPY receptors.

2.5 NPY binding to Y2 and Y5 reduces susceptibility to seizures

NPY is acting as an anticonvulsant by inhibiting glutamate release. However, which receptors are mediating this action is controversial. NPY receptors [Y1-Y5] are G protein-coupled receptors and they function to inhibit adenylyl cyclase, activate the mitogen-activated protein kinase (MAPK)-pathway, regulate intracellular calcium levels, and activate potassium channels. Currently, the role of the different receptors in seizure control is still controversial; however the NPY receptors that most likely play a suppressive role in NPY-related seizure control are Y2 and Y5. These receptors are most prominently expressed in the hippocampus (Redrobe et al., 1999) together with Y1. The role of Y2 and Y5 in NPY seizure control is studied mainly in experiments using agonists (Bijak, 1999;El Bahh et al., 2002;Woldbye and Kokaia, 2004;Nanobashvili et al., 2004) and antagonists (El Bahh et al., 2002;Nanobashvili et al., 2004;Benmaamar et al., 2005) against these receptors. However, a lack of highly selective agonists and antagonists for

the different NPY receptors subtypes makes it difficult to study the role these receptors separately (Lin et al., 2004;Woldbye et al., 2005). Furthermore, controversial data gained with agonist and antagonist can also be different due to using different seizure models (Woldbye et al., 2005).

To circumvent this problem experiments using Y2 and Y5 KO rodents are performed (Marsh et al., 1999;Baraban, 2002;Lin et al., 2004;Woldbye et al., 2005). Still, results are controversial and the role of Y2 and Y5 needs to be further examined. For instance, hippocampal slices from single Y2 and Y5 KO mice were less sensitive to seizures compared to slices of double KO mice (Woldbye et al., 2005). This indicates that both receptors regulate seizures *in vitro* and that these subtypes act in an adaptive and probably synergetic manner. However, Woldbye and co-workers also found that KA induced more severe seizures in Y5 KO mice, but not in Y2 KO mice, compared to wild type mice. This suggests that Y5 receptors reduce seizures *in vivo* and Y2 does not (Woldbye et al., 2005).

In addition, Kopp and colleagues measured the levels mRNA of NPY and various NPY receptors subtypes after experimentally induced seizures in rats (Kopp et al., 1999). They measured this in adult rat brains using in situ hybridization after 40 rapidly recurrent seizures induced with 5-min interval by hippocampal kindling stimulation. They found different expression levels before and after kindling, differences over time after kindling and differences in various brain regions and cell types. Reduced levels of Y2 mRNA after 2-4 h were found in the CA3 region and piriform cortex and increased levels of Y2 mRNA were found after 48 h and 1 week post-seizure in the dentate gyrus, amygdale and piriform and entorhinal cortices. The levels of Y5 mRNA were also increased, however this was only detected 2-5 h in the dentate granule cell layer and piriform and entorhinal cortices. This data indicate again that Y2 and Y5 probably reduce susceptibility to seizures at least in certain parts of the brain and as said before (section 2.3) probably changes in the expression of NPY and NPY receptors are altering the susceptibility to seizures and protect against recurrent seizures.

2.6. NPY binding to Y1 increases susceptibility to seizures

In the hippocampus, NPY receptor Y1 seems to have the opposite effect compared to Y2 and Y5. While Y2 and Y5 diminish seizures, NPY binding to Y1 facilitates seizures (Greber et al., 1994;Lin et al., 2004;Woldbye et al., 2005;Olesen et al., 2011). Using rAAV, overexpression of Y1 receptors was induced in the hippocampus of adult mice (Olesen et al. 2011). Kainate-induced seizures were aggravated in this model. This indicates that Y1 receptors increase seizure susceptibility. In addition, experiments performed with an Y1 antagonist suggested that the activation of the Y1 receptor was proconvulsant and blockade of Y1 receptors was anticonvulsant (Gariboldi et al., 1998) and both an agonist against Y2 and/or Y5 and an antagonist against Y1 reduced susceptibility to of seizures (Grundemar and Ekelund, 1996;Meurs et al., 2007).

Additionally, Y1 mRNA levels decrease in response to seizures in rodents (Kopp et al., 1999). This is the opposite effect of what was seen for receptors Y2 and Y5 (section 2.5). These data indicate that Y1 down-regulation and Y2 and Y5 up-regulation after a seizure decrease the susceptibility of NPY containing neurons and therefore the threshold for recurrent seizures is increased.

2.7 NPY and NPY receptors seem to play a different role in various brain areas

The hippocampus is a very important organ involved in epilepsy. However, more brain areas are known to be involved in seizure control. The role of NPY and NPY receptors seem to be different for various regions of the brain. For instance, the suppressive role of NPY and its receptors on seizures was observed in the frontal neocortex. NPY showed a suppressive effect on epileptiform discharges; however, results differed from those in hippocampus. The antiepileptic activity of NPY seems to be mediated by Y1 and not by Y2 (Bijak, 1999). This observation is the opposite of what was seen in the hippocampus. In the hippocampus, Y1 seems to increase the susceptibility to seizures.

In addition, as described in section 2.5 Kopp and co-workers found only up-regulation of Y2 and Y5 in specific brain parts and cells after seizures (Kopp et al., 1999). In other

brain regions they found reduced levels of Y2 mRNA. They also found that the mRNA expression of all receptors was time dependent. Probably, the mechanism of NPY-related seizure control is more complex then we have hypothesized so far.

2.8 Evidence for NPY-related seizure control from human studies

Up to now, most evidence has been found in studies using rodents. Nevertheless, there is also some data obtained by studying humans. In hippocampal specimens, obtained during autopsy from patients with TLE, Y2 receptor binding in the dentate hilus was increased by 43-48% (Furtinger et al., 2001). In addition, Y1 binding was significantly reduced by 62%. Moreover, intracellular recording from dentate granule cells, made in hippocampal slices from epileptic patients, showed a long-lasting inhibitory effect of NPY on excitation of neurons (Patrylo et al., 1999). Furthermore, patients with atypical febrile convulsions (FC) showed lower NPY plasma levels compared to patients with typical FC and controls (Lin et al., 2010). Atypical convulsions are seizures that persist for more than 15 minutes or that are recurrent within the same febrile period. Especially, very low concentrations of NPY were found in two patients; one patient suffered from seizures lasting for up to 1 hour and the other had many recurrent seizures during the same febrile illness.

2.9 Susceptibility to seizures is age dependent

Whether the function of NPY on seizure control is age dependent is an interesting question, besides other fascinating questions, that needs to be answered. That an older age increases the risk of getting epilepsy is seen in rodent and human studies. Also the role of NPY or the different subtypes of NPY receptors is studied in an age-related manner. Erickson and colleagues found that seizures usually occurred spontaneously in 6-8 weeks-old NPY-deficient mice (Erickson et al., 1996). Mice, older than 9 weeks, were more susceptible to seizures induced by the convulsant agent pentylenetetrazole (PTZ), a GABA antagonist. In these experiments 80% of NPY-deficient mice were susceptible to seizures compared to 33% of control mice. These results showed that NPY and age both play a role in seizure control.

Furthermore, it was suggested that older patients (more than a third of epileptic patients are over 65 years of age) are more susceptible to getting seizures because they have fewer NPY+ and paravalbumin (PV)+ neurons in their hippocampus. This hypothesis was examined by studying old (22-months) and young adult rats (5-months). KA induced acute seizures activity in older age rats and this was associated with far fewer hippocampal NPY+. The amount of the calcium binding protein PV+ interneurons was also reduced (Kuruba et al., 2011). Besides, plasma NPY levels were found to be significantly increased with age in FS patients (Lin et al., 2007).

In short, in different ways evidence has been found that NPY is involved in seizure control in rodents and probably also in humans. Increased levels of NPY reduce susceptibility of glutamate containing neurons and therefore high levels of NPY protect against seizures. In addition, various studies indicate that the up-regulation of Y2 and Y5 reduce susceptibility to seizures and that high levels of Y1 induce susceptibility to seizures. Likely, the up-regulation of NPY levels, Y2 and Y5 and the down-regulation of Y1 after a seizure protect against recurrent seizures. However, obtained data is somewhat controversial due to the lack of good agonist and antagonist, the use of different animal models and compensatory action of the receptors. Furthermore, the role of the NPY receptors subtypes seems to be time, brain region and cell type dependent.

3. The role of NPY in weight gain

Besides the role of NPY in seizure control, it is generally accepted that NPY is involved in energy homeostasis, a process that regulates food intake and energy expenditure. The process of energy homeostasis is a delicate balance and the disturbance of this balance may result in for instance malnutrition and an abnormal body shape (e.g. obesity, anorexia). Food intake and energy expenditure are dependent on various signals that makes these regulatory mechanisms very complex (Figure 2.) Internally, food intake is regulated by signals from adipose tissue, the gastrointestinal tract and from the brain. As well, external signals play a role. Energy expenditure, by physical activity and by heat production, is also linked with internal and external factors.

This chapter describes and discusses the role of NPY in energy homeostasis and especially in weight gain. Gaining weight is an unwanted side effect, because it is associated with several disorders as diabetes (Das and Mukhopadhyay, 2011) and cardiovascular problems (Aprahamian and Sam, 2011). It is interesting to elaborate the role of NPY in weight gain, because it has been shown that VPA affects both NPY and weight. Previously, the role of NPY in weight gain is already reviewed and summarized (Herzog, 2003;Huda et al., 2006;Valassi et al., 2008;Simpson et al., 2009;Zhang et al., 2011;Nguyen et al., 2011).

This thesis will summarize and discuss most important evidence for the role of NPY in weight gain. The role of NPY in weight gain has been examined for many years and in different ways. First, pharmacological approaches were used to study the role of NPY and NPY receptors. However, as mentioned before, the absence of specific agonist and antagonist for the individual NPY receptors makes it difficult to study the role of NPY in energy homeostasis by this route. Therefore, the role of NPY is studied extensively using transgenic animal models (summarized by (Herzog, 2003).

3.1 NPY suppression does not directly deregulate food intake

A couple of studies examined the effect of NPY inhibition on energy homeostasis (Erickson et al., 1996;Bannon et al., 2000;Yang et al., 2009). These studies found that inhibition of NPY does not directly lead to major changes. NPY deficient mice had a normal food intake; however, they became hyperphagic during food deprivation (Bannon et al., 2000;Erickson et al., 1996). In addition, NPY deficient mice were more sensitive to leptin (Erickson et al., 1996). They decreased their food intake more strongly and lost more weight than controls following leptin administration. This can be explained by the presence of compensatory mechanisms, for instance the up-regulation of NPY receptors.

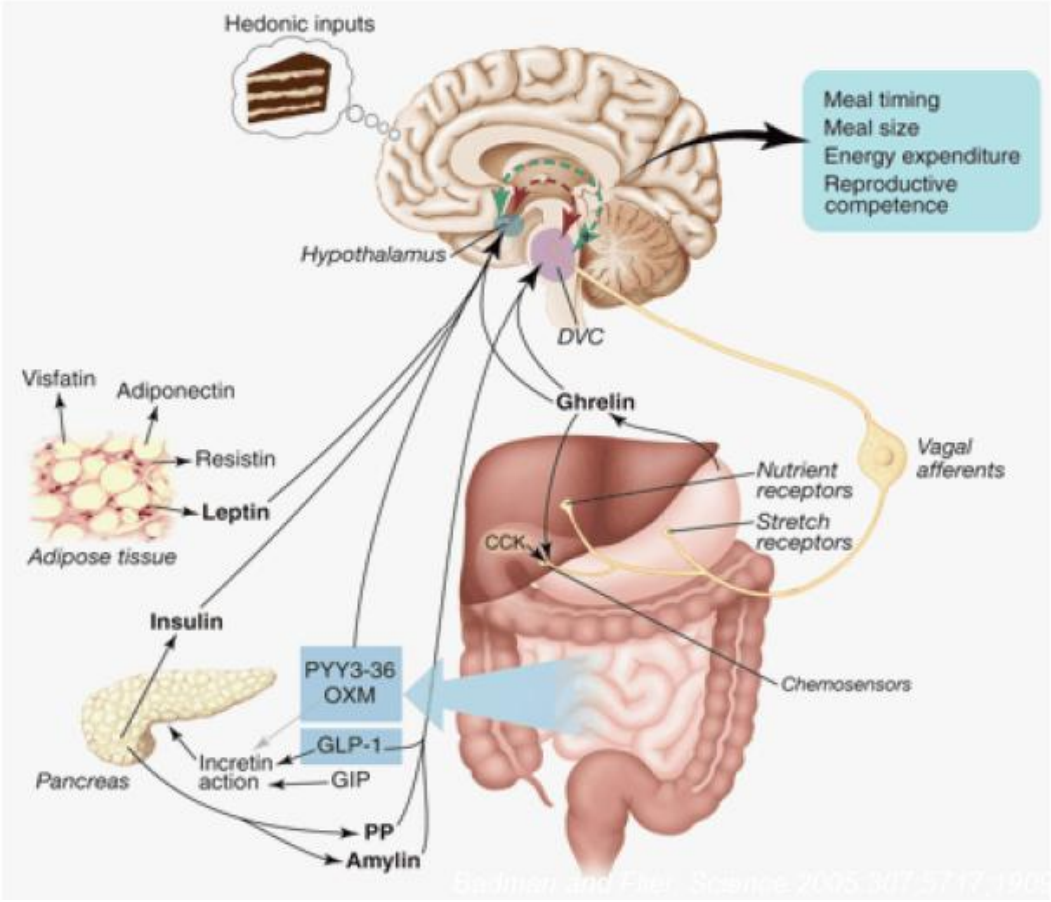

Figure 2: The complex mechanism of energy metabolism. Signals from the gastrointestinal tract, the pancreas and adipose tissue integrate collectively in the hypothalamus. Together these signals regulate meal timing, meal size, energy expenditure and reproductive competence. Important factors that act in this mechanism are e.g. ghrelin, leptin and insulin (Badman and Flier, 2005).

3.2 NPY overexpression increases appetite and result in weight gain

Besides NPY suppression, NPY overexpression was also examined by several research groups (Stanley and Leibowitz, 1985;Zarjevski et al., 1993;Sainsbury et al., 1996;Raposinho et al., 2001;Herzog, 2003;Lin et al., 2006;Yang et al., 2009). They all used only animal models in their study. From these experiments it became clear that increasing NPY in mice and rats leads to hyperphagia, body weight gain, hyperinsulinemia and other characteristics of the obesity syndrome. For example, the group of Stanley showed that chronic administration of NPY in rats in the lateral ventricle or directly in the paraventricular nucleus (PVN) leads to an increase in food intake followed by weight gain and ultimately obesity (Stanley and Leibowitz, 1985). Yang and colleagues found that AAV-mediated overexpression of NPY, in the dorsomedial hypothalamus (DMH) of lean rats, increases food intake and body weight (Yang et al., 2009). In this, weight gain was mainly induced by an increased food intake, because these rats were eating a more high fat diet.

3.3 Y2 and Y4 inhibit the action of NPY and Y1 and Y5 increase appetite

NPY may enhance appetite and thereby increase food intake. As described in the chapter about seizure control, various NPY receptors are involved in the action of NPY. Y2 and Y4 NPY receptors showed inhibitory effects on the action of NPY in energy homeostasis (Herzog, 2003). Y1 and Y5 seem to facilitate appetite. These results were also observed in studies using agonist and antagonists (Batterham et al., 2002;Henry et al., 2005;Sato et al., 2009;Shi et al., 2010). The roles of the individual NPY receptors seem rather different in seizure control and weight control. Y2 receptors decrease and Y1 receptors increase weight and susceptibility to seizures. However, Y5 showed a suppressive role in seizure control, but a stimulating role in energy homeostasis. More research is needed, though, to further determine the exact role of the different receptors, because published research is somewhat controversial.

3.4 Increased appetite and food intake via NPY is regulated in the hypothalamus

Whereas seizure control is primarily regulated in the hippocampus, appetite and food intake is mainly regulated in the hypothalamus. Figure 3 shows how energy metabolism is regulated in the arcuate nucleus (ARC), an aggregation of neurons inside the hypothalamus. Many hormonal and neuronal signals converge to the hypothalamus and especially to the ARC to modulate the response to nutrient ingestion (Ollmann et al., 1997;Batterham et al., 2002;Ellacott and Cone, 2004;Valassi et al., 2008;Simpson et al., 2009;Nguyen et al., 2011). The ARC contains two neuronal populations. These induce opposite effects. One population contains high amounts of the peptides NPY and agoutirelated peptide (AgRP) and these peptides stimulate food intake. The other population contains high amounts of the peptides pro-opiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART). These neuropeptides suppress food intake.

Similar to what has been observed for NPY and NPY receptors, neuropeptides like AgRP, POMC and CART are difficult to study due to counterbalancing actions. For instance, it was shown that hypothalamic POMC mRNA was increased in Y2-/-, ob/ob double KO mice (ob/ob is a mutant mouse in which both copies of *leptin* gene are mutated), but no effects on NPY, AgRP or CART concentrations were seen (Sainsbury et al., 2002).

The two neuronal populations in the ARC act on the PVN, the lateral hypothalamus (LHA) and perifornical area (PFA) (Figure 3). The PVN produces anorexigenic peptides (stimulating food intake) and the LHA and PFA are secreting orexigenic substances (suppressing food intake). The PVN is stimulated by NPY and AgRP and the LHA/PFA inhibit firing in response to these peptides. The neuropeptides POMC and CART induce the opposite effect.

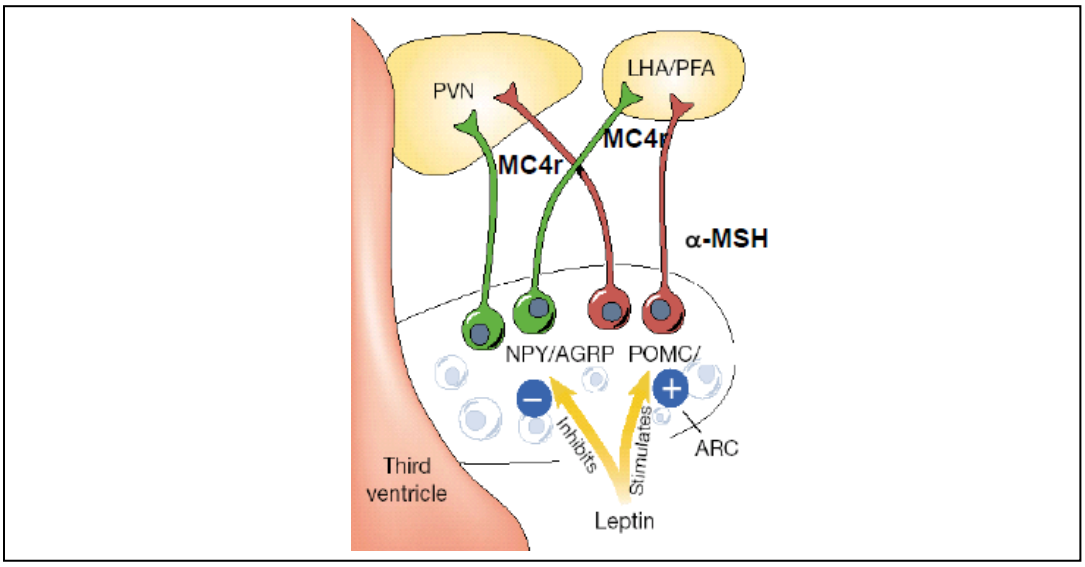

Figure 3: Regulation of energy homeostasis in the arcuate nucleus (ARC): NPY and AgRP containing neurons in the ARC enhance food intake and POMC containing neurons suppress food intake. Excitability of neurons is initiated by hormonal and neural signals e.g. leptin. α-MSH is converted from POMC and can activate mC4R in the PVN and the LHA/PFA (Schwartz et al., 2000).

3.5 NPY expression is affected by many hormonal and neuronal signals

As mentioned earlier, NPY is part of a complex mechanism in which many other factors (e.g. other transmitters, hormones, and nutrients) are involved. Important neuronal and hormonal signals that play a role in energy homeostasis are for instance satiety signals like cholecystokinin (CCK), glucogon-like peptide-1 (GLP-1) and peptide YY (PYY) originated from the gastrointestinal tract during meal. In addition, insulin secreted by pancreatic β-cells and leptin secreted by adipose tissue play an important role in energy homeostasis. These signals integrate in different parts of the hypothalamus (Figure 4) where they interfere with other signals. For instance, insulin and leptin are augmented during a positive energy balance and inhibit hypothalamic NPY expression (Schwartz et al., 1992;Ahima et al., 1996). Subsequently, this decreases appetite. Ghrelin induces the opposite effect of leptin and insulin (Asakawa et al., 2001;Shintani et al., 2001). Ghrelin stimulates ARC NPY/AgRP neurons and thereby NPY mRNA expression. This stimulates the induction of orexigenic signals from the PVN and therefore appetite is induced.

The opposite is also true. NPY can regulate the levels of other neurotransmitters and hormones. For instance, chronic NPY infusion in the lateral ventricle (LV) of the hypothalamus will eventually lead to an increase of insulin and leptin secretion (Raposinho et al., 2001).

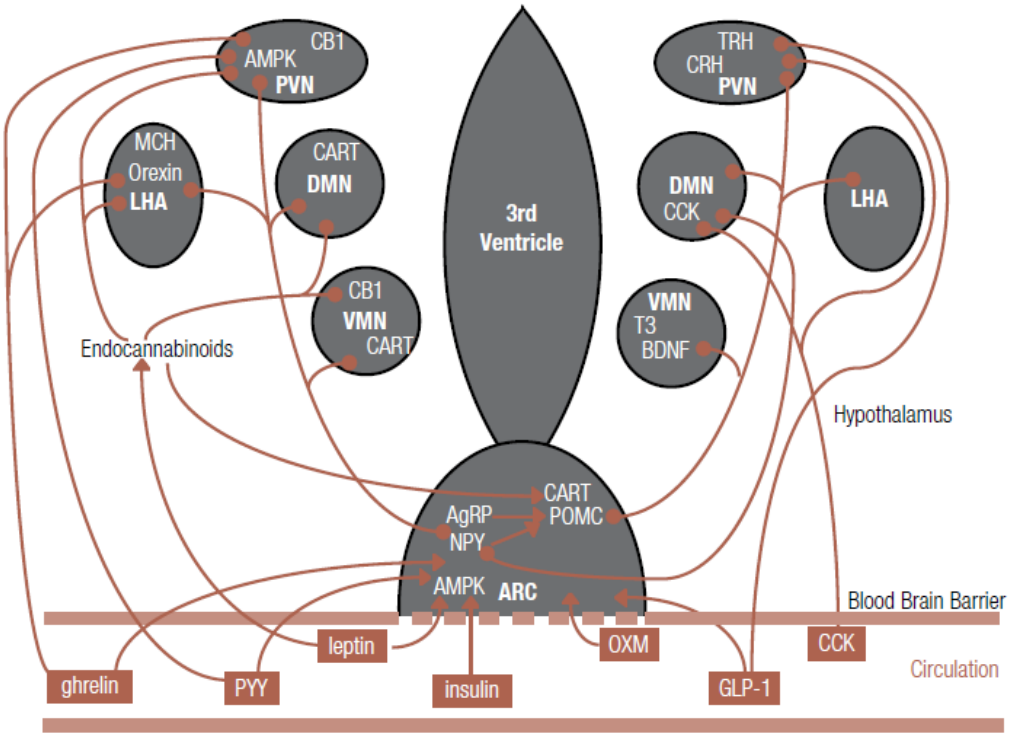

Figure 4: The regulation of energy homeostasis in the hypothalamus. Many signals integrate together in the ARC. Besided, hormones and neuronal peptides act on other areas in the hypothalamus and different hypothalamic areas are connected with each other. ARC = arcuate nucleus; PVN = paraventricular nucleus; VMN = ventromedial nucleus; DMN = dorsomedial nucleus; LHA = lateral hypothalamic area (Simpson et al., 2009).

In summary, NPY is a component of a complex mechanism, which is called energy homeostasis. In this balance, different factors affect each other. High levels of NPY induce appetite and therefore food intake. A chronic increase in appetite and food intake increases the risk of weight gain.

4. Is VPA-induced weight gain regulated via NPY?

Many studies have reported weight gain as a common side effect of VPA (Dinesen et al., 1984;Corman et al., 1997;Aydin et al., 2005;El-Khatib et al., 2007;Hamed et al., 2009;Martin et al., 2009;Cansu et al., 2011). This is concerning because it does not only affect the patients' body image and self-confidence; it also leads to pathologic consequences related to obesity, for instance hypertension, diabetes mellitus and atherosclerosis (Das and Mukhopadhyay, 2011;Aprahamian and Sam, 2011).

Although weight gain has been recognized as an adverse effect of VPA therapy the pathophysiology of VPA-associated weight problems remains unclear. In the first two chapters of this thesis the role of NPY in seizure control and energy homeostasis was described. In energy homeostasis NPY regulates food intake by increasing appetite (Das and Mukhopadhyay, 2011;Zhang et al., 2011;Nguyen et al., 2011). Besides, enhanced NPY levels reduce susceptibility to seizures (Erickson et al., 1996;Baraban et al., 1997;Richichi et al., 2004;Woldbye et al., 2005;Das and Mukhopadhyay, 2011). Because NPY plays a role in both mechanisms and by the fact that VPA enhances body weight, it is interesting to elaborate if VPA induces weight gain by affecting NPY.

4.1 NPY levels are raised after starting with VPA

Several studies have measured NPY levels after starting with VPA. These studies observed that NPY levels are indeed enhanced after starting with the drug (Aydin et al., 2005;Brill et al., 2006;Cansu et al., 2011). Whether VPA up-regulates NPY, directly or indirectly, is not apparent from these studies. Apart from NPY, other neuropeptides and hormones are up-regulated after starting with VPA. These neuropeptides are for instance insulin and leptin, also key players in energy homeostasis.

Aydin and colleagues found enhanced levels of NPY in all 20 epileptic patients after three months. In addition, they found an increase in insulin levels (Aydin et al., 2005). The concentration of leptin was only increased after 6 months, which indicate that an increase in leptin is most likely a secondary response. The group of Brill studied the

effect of VPA on NPY in rats (Brill et al., 2006). They found an increase in NPY levels in the nucleus reticularis thalami and the hippocampus in response to VPA already after 4 days of treatment. Cansu and colleagues also found an increase in leptin and NPY in epileptic children treated with VPA. However they examined their patients only after 18 months (Cansu et al., 2011).

Because energy homeostasis is a complex mechanism (Figure 2 and 4) it is difficult to discriminate on which factor VPA is acting directly. Changing one of the components involved in energy homeostasis, could deregulate the whole system and result in level changes of several peptides and lipids. Because energy homeostasis is such a complex mechanism, the action of VPA is probably multifactorial.

It would be interesting to study changes in the concentrations of peptides and hormones shortly after starting with VPA. There is one recent study that examined changes in feeding behaviors, energy expenditure and hormone levels (secreted in the gut) in healthy humans 3 weeks after starting the VPA treatment (Martin et al., 2009). In this study changes in weight gain, physical activity, levels of leptin, ghrelin, GLP-1 and PPY were measured and compared with a placebo group. They conclude that VPA-associated weight gain does not emerge to be the result of changes in activity levels or gut hormones; however they found an increase in food craving, hunger and binge eating. By studying changes shortly after starting with VPA we may possibly find the direct cause of VPA-induced weight gain. Levels of NPY were not measured in this study. Because it is known that NPY increases appetite, the increase in food craving, hunger and binge eating observed in this study could be a consequence of higher levels of NPY. An increase in appetite and food intake was observed in more studies (El-Khatib et al., 2007). However, there are also studies that show no changes in total energy intake, macronutrient selection and energy expenditure (Breum et al., 1992).

4.2. VPA could affect NPY via BDNF-mediate TrKB activation

One possible mechanism in which VPA might act on NPY could be that VPA affects NPY by inducing brain-derived neurotropic factor (BDNF). That BDNF enhances NPY is

shown *in vitro* (Barnea et al., 1995;Barnea et al., 2004) and *in vivo* (Croll et al., 1998;Reibel et al., 2003). Like NPY, BDNF has been shown to play a role in both seizure control and energy homeostasis. Chronic intrahippocampal induction of BDNF has been shown to delay kindling epileptogenesis most likely via NPY (Reibel et al., 2003). Besides, BDNF regulates homeostatic and hedonic feeding (feeding for pleasure) through a mechanism acting in the hypothalamus, dorsal vagal complex, and mesolimbic dopamine reward pathway (Cordeira and Rios, 2011).

BDNF activation of NPY is mediated by its receptor tyrosine-related kinase B (TrkB). Barnea and colleagues showed that BDNF leads to the phosphorylation of TrkB (Barnea et al., 2004). Furthermore, the up-regulation of NPY is dependent on the tyrosine kinase activity of TrkB and the blockade of TrkB receptors results in a decrease in basal *BDNF* mRNA levels (Wei et al., 2010). In addition, the extracellular singal-regulated kinase (Erk)-pathway, also called the MAPK-pathway, seems to be involved. VPA will probably activate BDNF via this pathway (Barnea et al., 2004). Probably, VPA activate the MAPK-pathway and this will subsequent lead to BDNF-mediated TrkB activation and finally to an increase in NPY expression.

This hypothesis is further supported by the results of Fukichi and colleagues (Fukuchi et al., 2009). Using microarray they found genes that were affected by stimulating cultured rat cortical neurons with VPA. *BDNF* was up-regulated in this experiment. Tang and colleagues compared expression patterns, with oligonucleotide microarrays, in the blood of epileptic patients treated with and without VPA. They observed changes in genes that are part of the MAPK-pathway (Tang et al., 2004).

The regulation of histone deacetylases (HDAC's) could be the way of action of VPA. HDAC's remove acetyl groups from histones. By acetylation, the positive charges on histones are neutralized and therefore DNA is bound less tightly and this results in more transcription. Thus, HDAC's prevent transcription. VPA is a powerful HDAC inhibitor (Gottlicher et al., 2001) and therefore VPA will induce more transcription for

instance the transcription of BDNF. Furthermore, it has been suggested that VPA enhances demethylation (Perisic et al., 2010). This also causes more transcription.

4.3 Rodents do not gain weight in response to VPA

More research is needed to examine if and how VPA induces weight gain via NPY. At present, these studies have not been performed. Human studies are probably the only way to elaborate this interesting question because mice (Chapman and Cutler, 1984) and rats (Wolden-Hanson et al., 1998) do not gain weight in response to VPA. VPA induces a significant increase in body weigh in female rhesus monkeys (Ferin et al., 2003), suggesting that the side effect weight gain may be characteristic of primate physiology.

It is not known why VPA does not induce weight gain in rodents. VPA treatment causes increased NPY expression in rodents. In addition, NPY overexpression induced experimentally causes increased appetite and finally weight gain. Furthermore, VPA suppress seizures in rodents. Probably the expression level of NPY is higher when it is induced experimentally then when it is induced by VPA. Furthermore, rodents can probably better control their energy homeostasis (food intake and energy expenditure) compared to primates in response to changes that influences this balance. Additionally, it is possible that VPA affects another factor that plays a role in the energy homeostasis of primates and not in rodents.

If we find out where these differences originate, we might find something that can help us by counteracting the negative side effects and promoting the positive effects of VPA.

5. Other mechanisms that could be involved in VPA-induced weight gain.

In the previous section, it has been described if and how VPA might induce weight gain via NPY. Because NPY is involved in both seizure and weight control it is possible that VPA directly acts on NPY and thereby increases weight in patients who use this medicine. Apart from this hypothesis, other hypotheses are described in the literature. These hypotheses are appropriately reviewed by a number of authors (Jallon and Picard, 2001;Hamed, 2007;Verrotti et al., 2011). In this section the most likely hypotheses are discussed.

NPY synthesis and secretion increases appetite and food intake. Probably, VPA is acting directly on NPY, however this remains currently uncertain. The up-regulation of NPY could also be a secondary response. For instance, it has been shown that insulin inhibits NPY syntheses and secretion in the PVN (Kalra et al., 1991). Thence, when a person becomes insulin resistant this person will probably have higher levels of NPY. Furthermore, leptin is able to down-regulate NPY (Jequier, 2002). In the literature insulin and leptin (and sensitivity for these hormones) are mentioned as candidate targets of VPA. Like for NPY, it is difficult to say if changes in insulin and leptin levels are a direct or indirect consequence of VPA.

5.1 An increase in leptin levels is most likely a secondary response of VPA

In various studies increased leptin levels were observed in response to VPA treatment (Aydin et al., 2005;El-Khatib et al., 2007;Hamed et al., 2009;Cansu et al., 2011;Grosso et al., 2011), which suggest a decreased sensitivity to leptin. Others found constant (Pylvanen et al., 2002;Pylvanen et al., 2006;Martin et al., 2009) or reduced leptin levels (Lagace et al., 2004). Aydin and colleagues measured an increase in the concentration of leptin only after 6 months (Aydin et al., 2005). In contrast, an increase in BMI, insulin, fasting insulin glucose ratio and NPY levels were already observed after 3 months of therapy. Moreover, leptin levels were observed to be increased in both epileptic patients with VPA-associated obesity and obese controls (Grosso et al., 2011). No changes in

leptin concentrations were observed in VPA-treated lean patients. Together, these observations imply that changes in leptin levels are induced by other metabolic alternations and not directly by VPA.

5.2. The occurrence of hyperinsulinemia and insulin resistance could be a direct consequence of VPA therapy.

Hyperinsulinemia and insulin resistance, in response to VPA, were observed in several studies (Pylvanen et al., 2002;Aydin et al., 2005;Pylvanen et al., 2006;Verrotti et al., 2009). Pylvanen and colleagues showed that VPA-treated patients had higher insulin levels compared to control subjects with similar BMI (Pylvanen et al., 2002;Pylvanen et al., 2006). Furthermore, both obese and lean VPA-treated patients developed insulin resistance. Aydin and colleagues determined an increase in hyperinsulinemia and insulin resistance already at the end of 3 months of treatment and these changes were seen with relatively low dosages of VPA (Aydin et al., 2005). In contrast, there are also studies that reported no insulin/insulin resistance after starting with VPA (Martin et al., 2009;Abaci et al., 2009;Cansu et al., 2011). These studies make it difficult to say something definite about the role of VPA on insulin/insulin resistance. However, insulin is a more likely target for VPA than leptin (Verrotti et al., 2011).

A couple of mechanisms are described that explain how VPA may increase weight via insulin/insulin resistance. (1.) VPA increases the concentration of long-chain fatty acids in patients their serum. This increase could be due to the structure of VPA as a fatty acid derivate. Fatty acids stimulate insulin secretion from pancreatic β-cells (Dobbins et al., 2002). Luef and colleagues showed that VPA can also stimulate insulin secretion from pancreatic β-cells (Luef et al., 2003). They found a direct effect of VPA on pancreatic island cells *in vitro* and VPA is acting on these cells in a dose and time dependent manner (Figure 5). Besides, VPA probably competes with free fatty acids (FFA) for albumin binding (Figure 6 (1)) (Vorum et al., 1993). Vorum and colleagues showed that the binding affinity for palmitate to serum albumin was decreased in VPA-treated patients. This results in an increased availability of long-chain fatty acids and again an increase in FFA stimulates insulin secretion from pancreatic β-cells. A chronic

increase in FFA will probably inhibit insulin release (lipotoxicity) (Yoshikawa et al., 2001). Nevertheless, these results make clear that an increase in FFA can disturb the balance of energy regulation. (2.) VPA may inhibit β-oxidation of fatty acids (Vorum et al., 1993). β-oxidation is a process in which FFA are broken down. This process occurs in the mitochondria. Fatty acids cannot pass directly through the mitochondrion membrane, but must be actively transported by a special transport system. A complex, consisting of three enzymes together with the co-enzyme carnitine, is responsible for this transport. VPA may interfere with carnitine, which leads to carnitine deficiency. When this occurs β-oxidation of fatty acids is inhibited (Figure 6 (2)). Inhibition of β-oxidation also leads to an increase in FFA availability in the patients their blood. In contrast, Demir and Aysun (2000) suggest that VPA no interfere with the enzyme carnitine (Demir and Aysun, 2000).

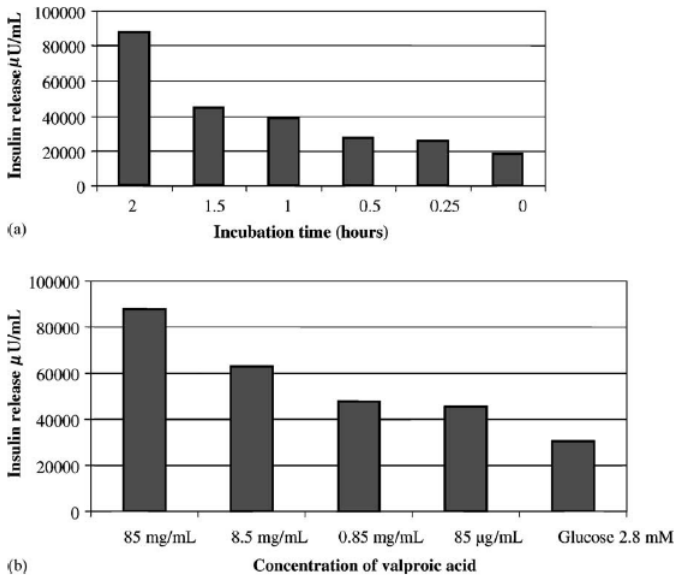

Figure 5: The effect of VPA on insulin release in pancreatic β-cells. (a) Various incubation times (b)Various concentrations of VPA.

Hyperinsulinemia, due to increased levels of FFA, will decrease lipolysis and increase lipogenesis (Vorum et al., 1993) (Figure 6 (3)). Subsequently, this results in lower blood glucose levels. This may explain why many authors report an increased food intake (Egger and Brett, 1981;Dinesen et al., 1984) since low glucose levels stimulate appetite.

That VPA induces hyperinsulinemia seems a plausible explanation. However, it must be kept in mind that high insulin and leptin concentrations in patients also may be the result of weight gain. Martin and colleagues showed no increase in insulin levels 3 weeks after starting the VPA treatment. In this study, weight was already increased with 0.49 kg (not significant)(Martin et al., 2009). This suggests that VPA does not directly act on insulin.

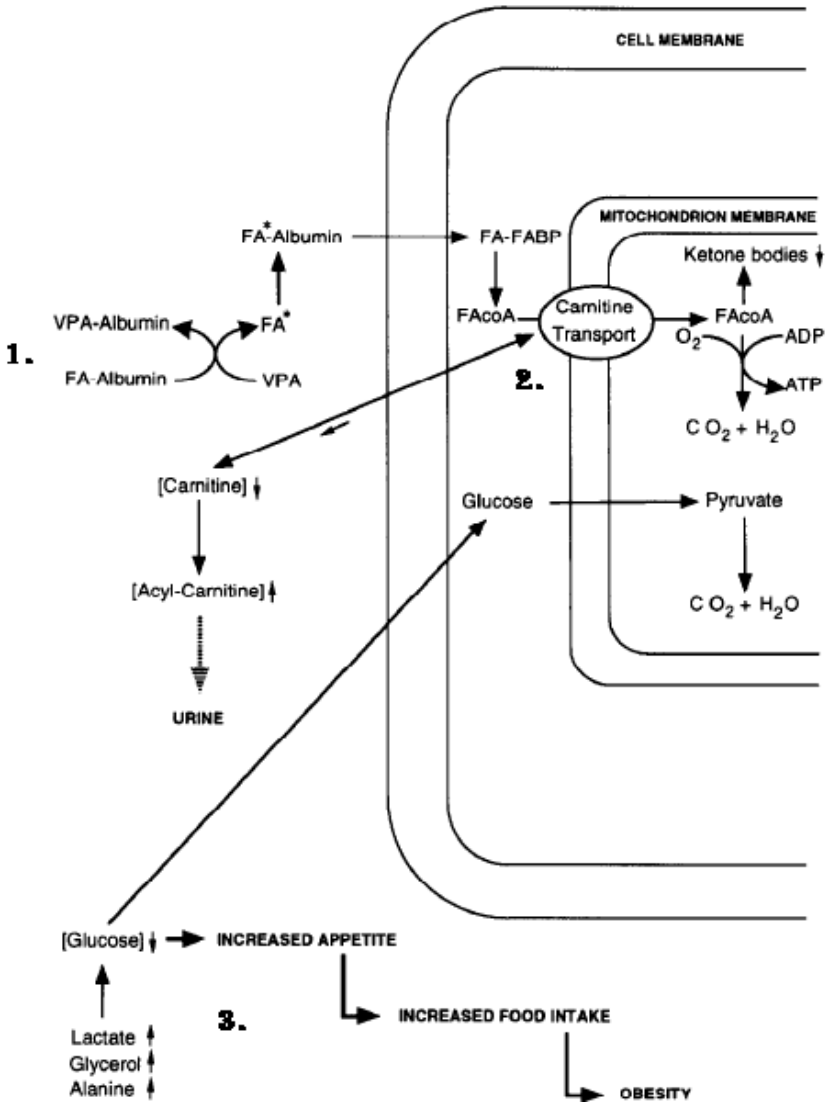

Figure 6: An increase of FFA by VPA increases food intake and may lead to obesity (Modified from (Vorum et al., 1993) (1) VPA probably competes with free fatty acids (FFA) for albumin binding. (2) VPA may interfere with carnitine which leads to carnitine deficiency and therefore β-oxidation of fatty acids is inhibited. (3) An increase in FFA results in lower glucose levels. Subsequently, this leads to an increase in appetite and food intake.

5.3 An effect of VPA on GABA is probably not the only mechanism of action

The neurotransmitter GABA could be a good candidate to be involved in VPA-induced weight gain (Jallon and Picard, 2001;Hamed et al., 2009). VPA is a GABA-ergic agonist and like NPY GABA is involved in both seizure control and energy homeostasis. In energy homeostasis GABA decreases food intake and blockade of the GABA receptor increases food intake (Panksepp et al., 1980). Furthermore, this neurotransmitter is known to be involved in pancreatic β-cell regulation and insulin secretion (Shi et al., 2000). Shi and colleagues showed that GABA functions as a negative regulator of insulin secretion in response to glucose by affecting K(ATP)(+) channels. Moreover, the plasma levels of GABA are found to be increased during VPA treatment (Johannessen, 2000). Thereby, VPA enhances GABAergic inhibition in various brain parts. On the other hand an effect on GABA is probably not the only mechanism of action, because there are more antiepileptic drugs that act on GABA, but that do not commonly induce weight gain (Hamed et al., 2009).

5.3 RSTN, FIAF and SOCS-3 are potentially target of VPA

Besides NPY and GABA, VPA acts probably on more neuropeptides in the hypothalamus. VPA seems capable of increasing *resistin (RSTN)* and *suppressor of cytokine signaling-3 (SOCS-3)* and decreasing *fasting-induced adipose factor (FIAF)* gene expression (Brown et al., 2007). The group of Brown described that this possibly occurred by decreasing the transcriptional activity of CCAAT-enhancer-binding protein(C/EBP)α. C/EBPs stimulate the expression of certain genes through interaction with their promoter. An altered expression of *RSTN, FIAF* and *SOCS-3* genes will develop insulin and leptin resistance (Howard and Flier, 2006). However the evidence that RSTN, FIAF and SOCS-1 interfere with insulin and leptin resistance is somewhat controversial (Lee et al., 2003). In addition, there is no evidence for this *in vivo*. Furthermore, as said for other factors it remains unresolved whether VPA directly or indirectly modifies gene transcription via a CEBPα-dependent mechanism or if this is a compensatory effect as a consequence of weight gain. It would be interesting to measure the increase and decrease of RSTN, FIAF and SOCS-3 after starting a VPA treatment.

5.4 VPA therapy increases the plasma levels of AP

Allopregnanolone (AP) can modulate synaptic and tonic inhibition, mediated by GABAa receptors, and the firing activity of 5-HT neurons (Grosso et al., 2003;Robichaud and Debonnel, 2006). In addition, it as been shown that AP has an evident hyperphagic effect in food-deprived mice. The group of Grosso examined the effect of VPA on AP levels (Grosso et al., 2011). They found higher AP levels in obese individuals whether or not under VPA therapy. Lean epileptic patients tended to have higher plasma AP levels than lean healthy controls, however this was not significant (p=0.06). It should be further studied (e.g. by studying larger groups) if an increase in AP in response to VPA is a direct or a secondary effect.

5.5 VPA therapy decreases adiponectin gene expression

Finally, VPA act probably on adiponectin. Qiao and co-workers examined the effect of VPA on *adiponectin* gene expression in mice and differentiated adipocytes (Qiao et al., 2006). They observed that VPA decreases *adiponectin* gene expression in mature adipocytes. This inhibitory effect was dose-and time dependent and occurred through the inhibition of HDAC activity. C/EBPα is down-regulated by inhibiting HDAC and therefore the binding of this transcription factor to the adiponectin promoter is inhibited. There is no clinical data concerning whether VPA affects adiponectin expression in human subjects. Adiponectin was shown to be impairing adiposite differention and increasing energy expenditure together with controlling insulin sensitivity and glucose homeostasis (Bauche et al., 2007).

In conclusion, many possible hypotheses are mentioned to explain VPA-induced weight gain. That VPA act directly on NPY could be true. However more peptides and lipids could be the main target of VPA, for instance insulin and adiponectin. Another interesting question is why not every patient, that uses VPA, suffer from weight gain. As described in section 2.9 the effect of VPA could be age dependent. In addition, it has been suggested that genetic factors are involved (Klein et al., 2005). Klein and colleagues investigated the effect of VPA on weight gain in monozygotic twins. They found that

VPA treated twins had a similar weight. This indicate that genetic factors may affect weight change caused by VPA. However, another good explanation could be that some patients have more discipline to control their weight. More research is needed to investigate these hypotheses in more details and as said in chapter 4 human studies will be important because rodents did not gain weight in response to VPA.

6. Conclusion

VPA is a drug that is used in many health problems such as bipolar disorders and epilepsy. That weight gain, induced by VPA, is a serious side effect is confirmed in many studies. Apart from its effect on patients' body image and self-confidence, weight gain may induce several pathological problems such as diabetes and hypertension. VPA has been available on the market for many years. It is therefore remarkable that the underlying mechanism of the working of this drug still needs to be defined. In addition, it is not known why VPA induces weight gain in humans.

Whereas the precise mechanism of VPA-induced weight gain needs to be further elucidated, some hypotheses are brought up. One of these hypotheses is that NPY is affected by VPA and that this may lead to a disturbed energy balance. It has been established that NPY plays a role in both seizure and weight control. In seizure control NPY decreases susceptibility to seizures. In energy homeostasis it increases appetite and thereby food intake. It has also been shown that NPY levels are increased after starting with VPA. Furthermore, an increased appetite is reported after starting the VPA-therapy. This makes NPY a possible target for VPA, though more research is needed.

Changes in NPY levels, after starting with VPA, should be measured in more patients. Current data were obtained by measuring NPY levels a couple of months after starting the therapy only. To find out if NPY is a direct target of VPA, levels of this neurotransmitter should be measured directly after starting the therapy. Furthermore, it should be further elaborated how VPA affects NPY. For instance, it would be interesting to determine which NPY receptors are most important.

The mechanism of NPY-related seizure control seems to be a complex process. The role of all receptors subtypes seems time, brain region and cell type dependent. Studies are underway to examine whether manipulation of NPY and NPY receptors can be used as treatment for epilepsies. If the effect on seizures and the effect on weight gain are coupled in humans, such treatment may have little success. However, if the effects are

uncoupled, such treatments may be promising. It should kept in mind that different brain areas will react differently in response to VPA. The hypothalamus is the most important organ regulating energy homeostasis and the hippocampus is the most important brain area involved in seizure control. Probably, the effect of VPA on seizure control and the effect on weight control could be uncoupled by manipulating NPY and or NPY receptors in different brain areas.

Moreover, there are several other hypotheses that explain VPA-induced weight gain. For instance, hyperinsulinemia and insulin resistance are suggested as principle mechanism underlying weight gain by VPA. In addition, other neurotransmitters such as *RSTN, FIAF* and *SOCS3* and hormones such as allopregnanolone and adiponectin could be affected by VPA. On the other hand, these factors do not play a role in seizure control like NPY.

Currently, physicians can only make patients aware of the possible side effect weight gain and help them with controlling their weight. By investigating the role of NPY in seizure control and weight gain, may possibly find a more effective therapy to treat epileptic patients without the unpleasant side effect weight gain.

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