Neuron-glia interactions in patients with type 2 diabetes

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Neuron-glia interactions in patients with type 2 diabetes

Martin Kalsbeek
NEURON-GLIA INTERACTIONS
IN PATIENTS WITH TYPE 2 DIABETES

Marten Jan Thomas Kalsbeek
Colophon

Neuron-glia interactions in patients with type 2 diabetes
Academic thesis, University of Amsterdam, the Netherlands

The research in this thesis was carried out at the
Laboratory of Endocrinology, Amsterdam University Medical Center (UMC), University of Amsterdam, Amsterdam Gastroenterology Metabolism, Amsterdam, The Netherlands.
Department of Endocrinology and Metabolism, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands.
Netherlands Institute for Neuroscience, an Institute of the Royal Netherlands Academy of Arts and Sciences, Amsterdam, the Netherlands.

About the cover
The cover represents the main experimental technique used in this thesis; the cutting of paraffin embedded human brain tissue into consecutive sections. These sections tend to stick together and form a ribbon, very similar to the negatives of a movie roll. The complex spectacle hiding inside these sections and negatives are revealed in the same way, with a simple beam of light. Some even say, the sound generated while cutting, sounds a lot like an old fashioned movie projector.

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NEURON-GLIA INTERACTIONS
IN PATIENTS WITH TYPE 2 DIABETES

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Introduction

Chapter 1

Based on

The infundibular peptidergic neurons and glia cells in overeating, obesity and diabetes.

Handbook of Clinical Neurology, Accepted.

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Abstract
Dysfunctional regulation of energy homeostasis results in increased bodyweight and obesity, eventually leading to type 2 diabetes mellitus (T2DM). The infundibular nucleus (IFN) of the hypothalamus is the main regulator of energy homeostasis. The peptidergic neurons and glia cells of the IFN receive metabolic cues concerning energy state of the body from the circulation. The IFN can monitor hormones like insulin and leptin, and nutrients like glucose and fatty acids. All these metabolic cues are integrated into an output signal regulating energy homeostasis through the release of neuropeptides. These neuropeptides are released in several inter- and extra-hypothalamic brain regions involved in regulation of energy homeostasis. This review will give an overview of the peripheral signals involved in the regulation of energy homeostasis, the peptidergic neurons and glial cells of the IFN, and will highlight the main intra-hypothalamic projection sites of the IFN.
General introduction
Obese individuals are at high risk of developing a variety of comorbidities including type 2 diabetes mellitus (T2DM). Losing this excess weight by intensive weight management showed a remission to a non-diabetic state in almost half of the individuals (Lean et al., 2018), indicating obesity causes T2DM. The excess weight in obesity results from an unbalance between energy intake and energy expenditure. While complex biologic systems regulate both energy intake and energy expenditure, the hypothalamus sits in the center of these systems controlling them. The crucial role of the hypothalamus in feeding behavior was first shown in the 1940’s, when hypothalamic lesions resulted in abnormal feeding behavior in rats (Brooks et al., 1946; Bertozzi, 1950). Since then, our understanding of the hypothalamic control of food intake and energy expenditure has expanded greatly, bulk of pharmacological and genetic approaches have revealed a variety of neuropeptides involved. Pharmaceutical companies spend tremendous efforts in developing therapeutics regulating neuropeptide function in order to combat obesity and T2DM, however so far with limited results.

The infundibular nucleus (IFN) is a brain region in the mediobasal part of the hypothalamus, situated adjacent to the third ventricle and above the median eminence (Krisch and Leonhardt, 1978; Broadwell et al., 1983; Shaver et al., 1992; Swaab, 2003). In this region metabolic cues from the periphery, like insulin and leptin, but also nutritional signals like glucose and fatty acids can penetrate the brain (Cottrell and Ferguson, 2004). The IFN integrates these signals into an output signal to second order neurons controlling energy homeostasis. In the IFN, two distinct populations of peptidergic neurons reside, which have opposing effects on energy homeostasis. The proopiomelanocortin (POMC) expressing neurons inhibit food intake and stimulate energy expenditure, whereas the agouti-related protein (AgRP) / neuropeptide Y (NPY) expressing neurons stimulate food intake and inhibit energy expenditure. Peptidergic neurons exert their effect mainly through the release of neuropeptides to second order neurons in inner-hypothalamic and extra-hypothalamic brain regions.

Furthermore, both neuronal populations in the IFN possess a variety of receptors for peripheral metabolic signals, but respond differently to the same stimuli. Next to the neuronal regulation of energy homeostasis, non-neuronal cells like astrocytes and microglia also play an active role in the control of metabolism (Freire-Regatillo et al., 2017). With their wide range of cell surface receptors, both cell types play an important role in the functional interactions of specific neuronal subpopulations (García-Cáceres et al., 2019).

This review will focus on the role of the main neuropeptides in the IFN in the human setting of feeding behavior, obesity and diabetes. First, we will give an overview of the peripheral signals that can affect the activity of infundibular peptidergic neurons and glial cells. Next, we will discuss the role of the different neuropeptides involved in food intake and energy expenditure. Last, we will highlight some of the main hypothalamic downstream projecting brain areas of the IFN.
Peripheral signals that influence the IFN

The IFN receives information about the energy status of the body in two ways, either directly through the sensing of available nutrients or indirectly through the action of hormones, which are produced in response to certain cues concerning energy intake or expenditure (Blouet and Schwartz, 2010). Some of these molecules are quite large, and how they pass the blood brain barrier (BBB) is still not completely clear (Banks, 2001; Murphy, 2015). However, the IFN locates right on top of the median eminence, which is devoid of a BBB (Rodriguez et al., 2010), and this lack of a BBB is thought to enable large molecules to passively diffuse and reach the IFN. Nevertheless, the IFN cells express receptors for glucose and fatty acids and they have receptors for the hormones insulin, leptin, ghrelin, peptide YY and glucagon-like peptide 1 (GLP1) (Perry and Wang, 2012; Timper and Brüning, 2017). All of these hormones and nutrients modify the IFN regulation of energy homeostasis; we will discuss the most important ones below.

Insulin

Insulin is probably best understood for its use as an anti-diabetic agent in the treatment of type 1 and type 2 diabetes mellitus (T1DM and T2DM), which is vital to maintain a “healthy” energy balance in these patients. In T1DM, the insulin producing β-cells of the pancreas are destroyed and very little to no insulin is produced. As insulin is vital for survival, these patients need to inject endogenous insulin on a daily basis to survive. In T2DM, the β-cells still function and can produce insulin, but long lasting episodes of hyperglycemic events have rendered the target cells insensitive to insulin, e.g. insulin resistant. These patients use high amounts of endogenous insulin or drugs that can increase insulin sensitivity to still provoke an insulin response and activate the downstream signaling pathways.

Insulin is the only glucose-lowering hormone known in the human body and the pancreatic β-cells release insulin in response to the postprandial rise of plasma glucose levels. Insulin then decreases plasma glucose through a whole pallet of actions in peripheral cells, like increased uptake of glucose into muscle and fat tissue, stimulation of glycogenesis and inhibition of gluconeogenesis (Petersen and Shulman, 2018). Next to these direct glucose-lowering properties in the periphery, central administration of insulin can indirectly lower glucose by inhibition of food intake (Woods et al., 1979). Furthermore, intranasal insulin was shown to decrease body fat in men, unfortunately not in women (Hallschmid et al., 2004).

Even though glucose uptake in the brain is thought to be independent of insulin signaling, insulin receptors (IRs) are widely expressed in the brain, especially in the hypothalamus (Havrankova et al., 1978). Both POMC neurons and AgRP/NPY neurons have been shown to express IRs on their cell surface (Benoit et al., 2002; Lam et al., 2017). The anorexigenic function of insulin is mediated by a stimulation of the POMC neurons and an inhibition of the AgRP/NPY neurons (Schwartz et al., 1992; Wang and Leibowitz, 1997; Havel et al., 2000; Benoit et al.,
Interestingly, while the ablation of all neuronal IRs resulted in obese mice (Bruning et al., 2000), the phenotype of mice lacking IRs specifically in POMC or AgRP neurons was unaltered (Konner et al., 2007). This indicated that insulin signaling in NPY neurons is crucial in the regulation of food intake and energy expenditure. Indeed, specific knock out of IRs on NPY neurons resulted in obesity in mice (Loh et al., 2017).

Astrocytes also express IRs, which play a supporting role in the glucose sensing mechanism of the IFN. Insulin signaling in astrocytes is important in the regulation of glucose uptake into the brain, ablation of IR in astrocytes reduces glucose-induced activation of POMC neurons and impairs physiologic responses to changes in glucose availability (García-Cáceres et al., 2016). The insulin receptor is also expressed on microglia, and the effect of insulin seems to be anti-inflammatory (Brabazon et al., 2018). Which is interesting in the control of energy homeostasis, as hypothalamic microglia activity has been shown to play a role in the dysfunctional regulation of energy homeostasis in diet-induced obesity, not only in rodents but also in humans (Thaler et al., 2012; Gao et al., 2014, 2017b, 2017a; Reis et al., 2015; Valdearcos et al., 2015; Baufeld et al., 2016; Yi et al., 2017).

**Leptin**

Adipocytes are the main site of leptin production and circulating leptin levels are highly correlated to body mass index (BMI) (Maffei et al., 1995). The function of leptin was discovered in the early 90s, when the leptin gene was identified (Zhang et al., 1994). The ob/ob and db/db mice do not produce leptin or leptin receptors (respectively), and both mutations result in a severely obese phenotype. Obese humans seem to be resistant to leptin as they have high levels of the circulating hormone but it fails to decrease food intake (Myers et al., 2010). In humans, congenital leptin deficiency is very rare, but it results in early onset obesity and can be treated with leptin substitution therapy (Funcke et al., 2014). The leptin molecule is quite large and how it crosses the BBB and reaches the neurons in the IFN remains unclear. Specialized ependymal cells in the median eminence called tanycytes have been proposed to play an important role in BBB transport of leptin (Langlet et al., 2013; Balland et al., 2014). It was also shown that this transport is still intact in obese mice, indicating leptin resistance is not due to impaired transport (Harrison et al., 2019). Even though the BBB transport of leptin is not completely known, it is clear that both POMC neurons and NPY/AgRP neurons express leptin receptors and they respond to leptin (Balthasar et al., 2004; Takahashi and Cone, 2005; Caron et al., 2018). Leptin stimulates POMC neuron activity and inhibits NPY/AgRP neurons, resulting in an anorexigenic effect.

Leptin can influence the neuronal control of energy homeostasis indirectly by its action on both astrocytes and microglia. Specific knockout of leptin receptor in astrocytes leads to altered feeding behavior; diminished leptin-induced anorexia and enhanced fasting- or ghrelin-induced hyperphagia (Kim et al., 2014). In microglia, specific leptin receptor knockout results in mice...
with higher body weight and hyperphagia with an associated decrease of POMC neurons (Gao et al., 2018).

**Gut hormones**

The brain also receives hormonal signals from the gastrointestinal (GI) tract; the GI tract produces these hormones mainly in relation to food ingestion. Ghrelin, also known as the hunger hormone, is produced by the stomach and stimulates feeding behavior (Tschop et al., 2000). Ghrelin levels are highest right before an anticipated meal, and the ingested meal suppresses ghrelin levels proportionately to the caloric content of the meal (Kirchner et al., 2012). Peripheral ghrelin administration induces feelings of hunger and increases food intake in lean and obese individuals. Ghrelin exerts its orexigenic effect through the IFN neurons, where it stimulates NPY/AgRP neurons and inhibits POMC neurons (Cowley et al., 2003). While ghrelin receptors are expressed by almost all NPY/AgRP neurons, only a small proportion of the POMC neurons express ghrelin receptors (Willesen et al., 1999). Additionally, ghrelin can mediate its metabolic action partly through astrocytes (Frago and Chowen, 2017). By changing astrocytic glucose and glutamate metabolism, ghrelin alters the levels of signals/nutrients reaching the neighboring neurons (Fuente-Martin et al., 2016). Furthermore, ghrelin has anti-inflammatory properties, which has also been shown in microglia (Theil et al., 2009).

Specialized enteroendocrine cells in the distal GI tract, called L-cells, produce peptide YY (PYY) in response to food ingestion. Intravenous infusion showed PYY inhibits food intake in both lean and obese individuals (Batterham et al., 2003). In the brain, PYY signals through the NPY receptors and in the IFN the Y2-receptor subtype is highly expressed (Batterham et al., 2002). The NPY/AgRP neurons express the Y2-subtype receptor and binding with PYY inhibits these neurons. Even though PYY was shown to stimulate POMC neurons, this does not seem to be essential for its anorexigenic effect, as PYY was shown to still inhibit food intake in MC4R and POMC knockout mice (Ghamari-Langroudi et al., 2005). When PYY is injected intracerebroventricular (ICV), it can bind to the Y1 and Y5 subtype receptor of second order neurons which results in a stimulatory effect on food intake, similar to NPY (Karra et al., 2009). Opposed to leptin, PYY levels are low in obese individuals and PYY infusion inhibits food intake (Batterham et al., 2003), which makes it interesting as an anti-obesity drug. However, the endogenous form of PYY has a short half-life, rendering it unpractical as a therapeutical. Several efforts to prolong the half-life of PYY have been performed and are still under examination (Hope et al., 2018).

The L-cells from the distal GI tract also produce glucagon-like peptide-1 (GLP-1), again in response to food ingestion (Holst, 2007). The release of GLP-1 has many peripheral effects including an increase of glucose stimulated insulin production (Jones et al., 2018). In the brain, GLP-1 directly activates POMC neurons and indirectly inhibits NPY/AgRP neurons, collectively inhibiting food intake (Baggio and Drucker, 2014). In overweight and obese
humans, treatment with GLP-1 receptor agonists showed to induce significant weight loss, irrespective of whether the patients had T2DM or not (Vilsboll et al., 2012), making it an interesting target as an anti-obesity drug.

**Nutrients**

The (unhealthy) western diet contains high amounts of sugar and fats, which are reflected in increased levels of these components in the general circulation, and the IFN can sense them directly. Glucokinase is a key enzyme in the neuronal glucose sensing machinery and it is expressed by both POMC and NPY/AgRP neurons (De Backer et al., 2016). POMC neurons are glucose responsive and upon stimulation they release α-MSH in a dose dependent manner (Parton et al., 2007). Furthermore, glucose sensing by POMC neurons became defective in diet-induced obese animals, which indicates neuronal glucose sensing plays a role in the development of T2DM (Parton et al., 2007). Glucose sensing neurons in the IFN can directly stimulate the pancreatic B-cells to secrete insulin (Rosario et al., 2016). NPY/AgRP neurons seem to be inhibited by glucose (Cheng et al., 2008). The glucose transporter 2 (GLUT2) is important for glucose sensing, and inhibition of GLUT2 mediated glucose-sensing increases food intake (Bady et al., 2006; Stolarczyk et al., 2010). Interestingly, GLUT2 signaling seems to be required specifically in astrocytes (Marty et al., 2005). Several studies have shown that astrocytes have an important role in glucose sensing in the IFN (Allard et al., 2014; Camandola, 2018). Fluctuating glucose levels have also been shown to activate microglia (Hsieh et al., 2019), Fatty acid signaling is mediated mainly by free fatty acid receptors (FFARs) (Falomir-Lockhart et al., 2019), and both neuronal populations in the IFN express FFARs (Dragano et al., 2017). The short chain fatty acid acetate can increase POMC and reduce AgRP expression in the hypothalamus (Frost et al., 2014), and the long chain fatty acids, oleic acid, increases POMC neuron excitability (Jo et al., 2009). Next to direct effects on IFN neurons, fatty acids influence the immune response in the hypothalamus, indirectly affecting central control of energy homeostasis. Several studies have shown that fatty acids can either cause or attenuate hypothalamic inflammation in diet-induced obesity (Mendes et al., 2018).

Amino acid levels are also a cue of nutrient availability and in the periphery cells use mTOR signaling to sense amino acids (Goberdhan et al., 2016). In the IFN, mTOR signaling has been shown to play an active role in the regulation of food intake and can be activated by the amino acid L-leucine (Cota et al., 2006). While it seems clear that the amino acid leucine is sensed by the brain, its role in food intake remains controversial (Zampieri et al., 2013; Laeger et al., 2014; Maurin et al., 2014).

**Neuropeptides and their receptors**

Melanocortins are peptide hormones derived from POMC, and induce their effect through the melanocortin receptors (MCRs). Over two centuries ago, it was already discovered that mutations
in the POMC gene are associated to early onset obesity and severe overeating (Krude et al., 1998). POMC is a pre-cursor polypeptide, which undergoes post-translational modifications and can give rise to the signal peptides α, β and γ-melanocyte stimulating hormone (MSH), adrenocorticotropic hormone (ACTH) and β-endorphin. The main melanocortin related to food intake is α-MSH, which inhibits food intake. Injection of an α-MSH analog into the third ventricle inhibits food intake in food deprived rats (Brown et al., 1998). In humans, the expression of α-MSH in post-mortem brain tissue did not correlate to BMI, but was significantly decreased in diabetic patients (Alkemade et al., 2012). Next to the effect on neuronal control of food intake, α-MSH also has an anti-inflammatory effect (Delgado et al., 1998). Both astrocytes and microglia express the MCRs and activation of this receptor results in decreased inflammation in several different ways (Carniglia et al., 2013; Caruso et al., 2013).

There are five MCRs and the brain only harbors two of them; MC3R and MC4R. Of these two melanocortin receptors, the role of MC4R in energy homeostasis has historically received the most scientific attention. Actually, MC4R deficiency is the most common monogenic form of obesity (Farooqi et al., 2003) and several studies have shown MC4R mutations in obesity (Hinney et al., 1999; Farooqi et al., 2000; Vaisse et al., 2000). Additionally, a huge genome wide association study revealed over 50 new candidate genes associated with BMI, most of them are enriched in the CNS and many of them belong to the melanocortin system, specifically the MC4R (Locke et al., 2015). The MC4Rs are widely expressed throughout the human hypothalamus, with highest expression in the paraventricular nucleus (PVN), supra optic nucleus (SON) and the nucleus basalis of Meynert (NBM) (Siljee et al., 2013). Researchers studied the role of MC4R in the PVN intensively and showed it to be important for feeding behavior (Cowley et al., 1999; Atasoy et al., 2012). MC4R knockout mice have an obese phenotype characterized with hyperphagia and increased adiposity (Butler et al., 2001), which underscores the importance of MC4R in healthy energy homeostasis. Surprisingly, since their discovery, nobody investigated the role of MC4Rs in the SON and NBM and their function therefor remains unclear.

Opposed to MC4R, mutations in MC3R gene did not show up in an association to obesity (Calton et al., 2009) and MC3R knockout studies did not show a clear phenotype (they display moderate adiposity without being hyperphagic) (Butler et al., 2000; Chen et al., 2000). This probably explains the historic lack of attention for the role of MC3R in energy homeostasis. But in more recent years, research into the role of MC3R has increased and a systematic review showed that loss of function mutations in the MC3R gene confers a three-times increased risk of obesity in humans (Ehtesham et al., 2019).

Another neuropeptide cleaved from the precursor POMC is β-endorphin, which is not considered to be a melanocortin because it uses the µ-opioid receptor. It does, however, affect the central control of food intake (Appleyard et al., 2003). The acute effect of β-endorphin seems to be the stimulation of food intake by antagonizing the effect of α-MSH (Dutia et al., 2012).
However, under chronic conditions this increase in food intake was not sustained (Dutia et al., 2012). Transgenic mice lacking β-endorphin, but with normal melanocortin signaling, became hyperphagic and obese, indicating β-endorphin has an inhibitory effect on food intake (Appleyard et al., 2003). It has also been suggested that β-endorphin plays a motivational role in feeding behaviour (Veening and Barendregt, 2015).

**Agouti related Protein**

AgRP and its related protein agouti are the only known endogenous reverse agonists of the melanocortin receptors. By decreasing activity of MCR expressing cells, AgRP can decrease the inhibition of food intake, effectively increasing food intake. In animal studies, AgRP is a strong appetite inducer and results in hyperphagia and the development of obesity when transgenically overexpressed or when administered ICV. In humans, however, little is known about mutations in the AgRP gene that correlate to human obesity or diabetes. One study investigated a mutation which induced enhanced promotor activity of the AgRP gene and found that this mutation was increased in Siera Leonians with a “high BMI” (a BMI above the mean BMI), compared to individuals with a “normal BMI” (a BMI below the mean) (Mayfield et al., 2001). In anorectic patients, they found an increase of mutations in the AgRP gene possibly affecting receptor binding, indicating that a decreased affinity of AgRP protein for the MCR results in decreased food intake (Vink et al., 2001).

**Neuropeptide Y**

Even though 90% of the NPY neurons co-express AgRP, the neuropeptide NPY itself does not bind to melanocortin receptors. There are six NPY receptors known and three of them are highly expressed in the brain (Y1, Y2 and Y5). Animal studies revealed NPY as a potent stimulator of food intake; chronic infusion of NPY led to hyperphagia and obesity (Beck et al., 1992) and ICV injections of NPY stimulated food intake (Levine and Morley, 1984). NPY in the brain in mainly produced by the IFN neurons (Chronwall et al., 1985) and it elicits consumptive behavior through several second order neuronal nuclei, like the PVN, VMH and LH (Stanley et al., 1985). NPY neurons express insulin and leptin receptors and both hormones inhibit NPY expression (Jang et al., 2000; Loh et al., 2017), ghrelin has the opposite effect and stimulates NPY expression (Hashiguchi et al., 2017).

In non-diabetic humans the expression of NPY shows a negative correlation with BMI (Alkemade et al., 2012) and obese individuals have significantly less NPY expression compared to lean controls (Goldstone et al., 2002). Conversely, T2DM subjects have significantly increased NPY expression compared to non-diabetic controls (Saderi et al., 2012). Together, these studies indicate that there is a negative feedback of adiposity on NPY expression, which is lost in T2DM. As leptin is the main signal of adiposity, it seems likely that NPY neurons in T2DM have become leptin resistant.
Five different Y-receptors have been identified, of which Y1, Y2 and Y5 have been implicated in the central regulation of energy homeostasis. Both Y1 and Y5 receptors are mainly expressed on second order neurons and have been shown to mediate NPY-induced hyperphagia (Nguyen et al., 2012). Conversely, Y2 receptors are mainly found on NPY neurons themselves and activation by NPY or PYY results in decreased neurotransmitter release (Yulyaningsih et al., 2011). Thus, stimulation of the Y2 receptor on NPY neurons leads to inhibition of food intake, which has fueled the development of Y2 agonists (Feletou et al., 2006).

**Second order neurons**

Upon activation by peripheral signals, the IFN neurons release their neuropeptides to several second order neurons in different brain areas to exert their control on energy homeostasis. The main hypothalamic projection sites are the paraventricular nucleus (PVN), the ventromedial hypothalamus (VMH) and the lateral hypothalamus (LH).

**Paraventricular nucleus**

The PVN combines the metabolic input from the IFN, with input from many other hypothalamic regions, like information concerning circadian rhythms from the suprachiasmatic nucleus (Buijs et al., 1998). The PVN consists of two major sections, the parvocellular and magnocellular division (Swanson and Kuypers, 1980). The parvocellular neurons project to the autonomic nervous system, thereby linking the PVN to all the major peripheral organs involved in energy homeostasis (Hill, 2012). The magnocellular neurons project to the pituitary, where they can release oxytocin and vasopressin into the systemic circulation. Vasopressin in the circulation increases water reabsorption in the kidneys and constrict arterioles to increase blood pressure. In the brain, activation of vasopressin neurons can inhibit food intake (Yoshimura et al., 2017). Oxytocin, also known as the love-hormone, has many functions, including a role in social bonding, childbirth and sexual reproduction. In the brain, oxytocin signaling has a role in feeding behavior, and intranasal application of oxytocin was shown to decrease food intake in humans (Ott et al., 2013; Lawson et al., 2015). In general, activation of the PVN neurons decreases food intake, which was first shown by lesions in the PVN which resulted in hyperphagic and obese rats (Leibowitz et al., 1981). The neurons in the PVN express melanocortin receptors (Siljee et al., 2013) and Y-receptors (Yoshimura et al., 2017). Both neuropeptide receptors in the PVN have been shown to be involved in the control of energy homeostasis (Reichenbach et al., 2012; Shi et al., 2013).

**Ventromedial hypothalamus**

The role of the VMH in feeding behavior was the topic of discussion in the early 70s (Gold, 1973), but we now know that the VMH can be classified as the satiety center (King, 2006). The VMH neurons express NPY receptors and NPY inhibits the activity of VMH neurons, which increases food intake (Chee et al., 2010). The neurons in the VMH are also leptin responsive,
and leptin infusion in the fourth ventricle results in VMH-mediated weight loss (Seamon et al., 2019). The VMH neurons play a role in the regulation of glucose homeostasis. Activation of a specific subset of VMH neurons by designer receptor exclusively activated by designer drugs (DREADD), increased insulin sensitivity and depressed glucose production (Coutinho et al., 2017). Whereas, optogenetic stimulation of a (different) subset of VMH neurons caused diabetes-range hyperglycemia (Meek et al., 2016).

**Lateral hypothalamus**

The LH is generally known as the hunger center, and two of its main functions are the stimulation of feeding behaviour and arousal. Electrical stimulation of the LH results in ravenous eating behaviour, and animals are extremely motivated to work for a food reward (Stuber and Wise, 2016). The neurons of the LH are mainly orexin expressing neurons and they respond to both melanocortins and NPY (Campbell et al., 2003; Morgan et al., 2015). Orexin neurons stimulate wakefulness, and a loss of orexin neurons causes narcolepsy (Shan et al., 2015), which in turn is associated to increased risk on the development of T2DM (Mohammadi et al., 2019). Together, these properties of orexin promotes alertness in a fasted state, which is crucial for food seeking behavior (Tsujino and Sakurai, 2013).

**Concluding remarks**

The regulation of energy homeostasis is a complex interplay between numerous peripheral and central biological systems. Several endogenous and exogenous factors can deregulate energy homeostasis, which, in combination with a western diet, usually results in a positive energy balance. Long-term positive energy balance eventually leads to obesity and prolonged obesity often results in T2DM. This review focused mainly on the control of energy homeostasis itself, as flawed control is the key underlying factor in the development of obesity and T2DM. A large part of the research listed in this review was obtained through animal studies, which is not always directly translatable to human pathophysiology. For example, it is known that animal models do not completely mimic the human situation of T2DM development, unless a genetic or pharmacologic intervention is performed. Rodents that are fed with only an obesogenic diet do become obese and hyperglycemic, but do not develop T2DM with pancreatic β-cell failure like humans do. On the other hand, human *in vivo* data lack satisfactory spatio-temporal resolution to study specific cell types or molecules. Studies using human post-mortem brain tissue do provide the possibility to study cell types and molecules, but only allow for association studies. With all these limits in animal and human studies, it is not surprising that some successful anti-obesity pharmacological interventions in animal models proofed to have no effect in humans. Collectively, this review gives an overview of the latest human and animal data concerning the IFN control of energy homeostasis, which posses several targets in the combat against obesity and T2DM.
References


References


García-Cáceres, C., Quarta, C., Varela, L., Gao, Y., Gruber, T., Legutko, B., Jastrech, M.,
Kim, J.G., Suyama, S., Koch, M., Jin, S., Argente-Arizon, P., Argente, J., Liu, Z.-W., 
Zimmer, M.R., Jeong, J.K., Szigeti-Buck, K., Gao, Y., Garcia-Caceres, C., Yi, C.-X., 
Salmaso, N., Vaccarino, F.M., Chowen, J., Diano, S., Dietrich, M.O., Tschop, M.H., 
Horvath, T.L., (2014). Leptin signaling in astrocytes regulates hypothalamic neuronal 
King, B.M., (2006). The rise, fall, and resurrection of the ventromedial hypothalamus in the 
regulation of feeding behavior and body weight. Physiol. Behav. 87, 221–244.
Kirchner, H., Heppner, K.M., Tschop, M.H., (2012). The role of ghrelin in the control of 
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.
ergy-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC 
Laeger, T., Reed, S.D., Henagan, T.M., Fernandez, D.H., Taghavi, M., Addington, A., 
brain to suppress food intake but does not function as a physiological signal of low 
Lam, B.Y.H., Cimino, I., Polex-Wolf, J., Nicole Kohnke, S., Rimmington, D., Iyemere, 
V., Heeley, N., Cossetti, C., Schulte, R., Saraiva, L.R., Logan, D.W., Blouet, C., 
O’Rahilly, S., Coll, A.P., Yeo, G.S.H., (2017). Heterogeneity of hypothalamic pro-
opiomelanocortin-expressing neurons revealed by single-cell RNA sequencing. Mol. 
Metab. 6, 383–392.
Langlet, F., Levin, B.E., Luquet, S., Mazzone, M., Messina, A., Dunn-Meynell, A.A., 
Ballard, E., Lacombe, A., Mazur, D., Carmeliet, P., Bouret, S.G., Prevot, V., Dehouck, 
B., (2013). Tanyctytic VEGF-A Boosts Blood-Hypothalamus Barrier Plasticity and 
Access of Metabolic Signals to the Arcuate Nucleus in Response to Fasting. Cell Metab. 
17, 607–617.
for remission of type 2 diabetes (DiRECT): an open-label, cluster-randomised trial. 
lesions produce overeating and obesity in the rat. Physiol. Behav. 27, 1031–1040.
Locke, A.E., Kahali, B., (2015). Genetic studies of body mass index yield new insights for 
Loh, K., Zhang, L., Brandon, A., Wang, Q., Begg, D., Qi, Y., Fu, M., Kulkarni, R., Teo, 
controls food intake and energy balance via NPY neurons. Mol. Metab. 6, 574–584.


Scope of the thesis

The overall aim of this thesis is to understand how neurons and glial cells are affected in the brain of metabolic disordered humans. The infundibular nucleus (IFN) of the hypothalamus – the so-called window of the the brain, is the main regulator of food intake, glucose regulation and overall whole body energy homeostasis. A variety of peptidergic neurons and glial cells in the IFN receive metabolic feedback concerning the peripheral energy state of the body. All these metabolic cues are integrated into an output signal that regulates energy homeostasis through the release of neuropeptides, neurotransmitters and neuromodulators in several second order inter- and extra-hypothalamic brain regions. In Chapter 1, we provide an overview of the peripheral signals involved in the regulation of energy homeostasis, the peptidergic neurons and glial cells of the IFN, and highlight the main intra-hypothalamic projection sites of the IFN. Previous research has clearly established an important role for the hypothalamic IFN in the regulation of energy homeostasis during the development of obesity and type 2 diabetes (T2DM). However, the large majority of this work has been done in experimental animals, but none of these animal models is able to completely mimic human T2DM pathophysiology. Furthermore, in daily life humans are exposed to a large variety of environmental factors, while in laboratory animals these environmental factors are much more limited. On top of that, humans receive treatment for their disease, in this case anti-diabetic drugs, which most likely will change the disease pathology. Therefore, we performed a series of studies using post-mortem brain tissue, to explore human brain dysfunction in T2DM, using the unique collection of human brain material from the Dutch human brain bank. In Chapter 2, we studied how the different anti-diabetic treatments affect the peptidergic neurons and glia cells in the IFN. In Chapter 3, we investigated whether chronic T2DM pathogenesis is linked to a dysfunction of the biological clock machinery that is located in the human suprachiasmatic nucleus (SCN). In Chapter 4, we explored the mechanistic link between T2DM and the progression of Alzheimer’s disease (AD), by examining the cholinergic neurons (using acetylcholine transferase as a marker), hyperphosphorylated tau that is expressed at different stages of cognitive decline and the microglial cells in the nucleus basalis of Meynert (NBM).

Over the last decade, many studies have discovered that hypothalamic inflammation is associated with the CNS pathophysiology of diet-induced obesity, insulin resistance and diabetes. It also has become clear that microglia immunometabolism plays a critical role in controlling microglial immune activity and the inflammatory responses in the micro-environment of the hypothalamus. We know that a hypercaloric diet will activate microglia, but which components of the hypercaloric diet are (most) responsible is not well known. Microglia can use different energy substrates, but fuel influx from the different substrates is also determined by microglial
activity. Little is known about how microglial functionality and cellular metabolism are integrated. In Chapter 5, we summarized the most recent data from human, mouse and rat studies that are relevant for energy metabolism of microglia, to provide an overview of the pathways that are important for microglia metabolism under different physiological and pathophysiological conditions. Previous studies also provided evidence for the existence of a daily rhythmicity in microglial cells, with higher activity during the dark (i.e. the active phase in rodents), and an attenuation of this rhythmicity during chronic hypercaloric conditions. In chapter 6, we confirmed the deleterious effect of a hypercaloric environment on microglial immunometabolism and its daily rhythmicity.
Chapter 2

The impact of anti-diabetic treatment on human hypothalamic infundibular neurons and microglia

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Abstract
Animal research indicates that hypothalamic dysfunction plays a major role in the development of type 2 diabetes mellitus (T2DM), insulin resistance and inflammation are important mechanisms involved in this disorder. However, how T2DM and anti-diabetic treatments affect the human hypothalamus is still far from clear. Here we characterized the proopiomelanocortin (POMC) immunoreactive (-ir) neurons, the neuropeptide-Y (NPY)-ir neurons, the ionized calcium-binding adapter molecule 1 (iba1)-ir microglia and the transmembrane protein 119 immunoreactivity (TMEM119)-ir microglia in the infundibular nucleus (IFN) of post-mortem human hypothalami of 32 T2DM subjects with different anti-diabetic treatments and 17 matched non-diabetic control subjects. Comparing T2DM subjects to the matched control subjects showed a decrease in the number of POMC-ir neurons, but no changes in NPY-ir neurons or microglia. Interestingly, T2DM subjects treated with the anti-diabetic drug metformin had less NPY-ir neurons and less microglia compared to T2DM subjects without metformin treatment. Finally, we found that the number of microglia correlated with the number of NPY-ir neurons, but only in T2DM subjects. These results indicate that different changes in POMC and NPY neurons and microglial cells in the IFN accompany T2DM. In addition, T2DM treatment modality is associated with highly selective changes in hypothalamic neurons and microglial cells.
Introduction

Hypothalamic dysfunction is known to play a major role in the development of obesity and type 2 diabetes (T2DM) (Morton, Cummings et al. 2006, Begg and Woods 2013). A vast number of studies have focused on the hypothalamic infundibular nucleus (IFN, equivalent to the arcuate nucleus in rodents), which contains two key neuronal populations regulating energy homeostasis, proopiomelanocortin (POMC) expressing and neuropeptide Y (NPY) / agouti-related protein (AgRP) expressing neurons. Diet-induced obesity and T2DM have been associated with a disturbed balance between the anorexigenic POMC (Alkemade, Yi et al. 2012, Thaler, Yi et al. 2012) and the orexigenic NPY/AgRP neurons (Saderi, Salgado-Delgado et al. 2012, Zhang, Yang et al. 2016).

In recent years, animal studies have indicated hypothalamic inflammation as an important player in the changes in hypothalamic neuronal function observed during the development of obesity and T2DM (Arruda, Milanski et al. 2011, Cai 2012, Milanski, Arruda et al. 2012, Pimentel, Ganeshan et al. 2014). In these studies, hypothalamic inflammation was demonstrated by increased expression of pro-inflammatory factors mainly produced by reactive microglia, the resident innate immune cells of the brain. Although interactions between hypothalamic neurons and microglia are apparent in animal models (Valdearcos, Xu et al. 2015, Gao, Layritz et al. 2017, Yi, Walter et al. 2017), the clinical relevance of these findings in the pathophysiology of human T2DM is completely unknown.

The diagnosis of T2DM in humans is based on elevated fasting plasma glucose levels, and anti-diabetic treatments aimed to lower glucose levels. Treatment entails a stepwise approach consisting of lifestyle changes and sequential addition of oral anti-diabetics (standardly metformin) and insulin if necessary (Bouma, Rutten et al. 2006). The insulin sensitizer metformin lowers blood glucose levels via suppressing hepatic glucose production and increasing skeletal muscle glucose clearance (Giannarelli, Aragona et al. 2003). Metformin is known to pass the blood brain barrier and affect the expression of hypothalamic neuropeptides in diabetic rats (Lv, Wen et al. 2012), and decrease NPY gene expression in cultured hypothalamic neurons (Chau-Van, Gamba et al. 2007). Likewise, also insulin is well known to affect neuropeptide expression in the rodent hypothalamus (Schwartz, Sipols et al. 1992, Benoit, Air et al. 2002). Interestingly, in addition to their effect on neurons, both metformin and insulin also have anti-inflammatory properties (Sun, Li et al. 2014, Saisho 2015). These data indicate that both oral anti-diabetics and insulin analogues might not only influence hypothalamic neurons, but also hypothalamic microglia.

In the current study we aimed to answer the following questions: are NPY and POMC neuronal numbers altered in T2DM? Is soma size, as a functional measure of the structural integrity and viability of neurons, altered in T2DM? Is microglial activity, as reflected by soma number, size and ramification in the IFN associated to (changes in) NPY and POMC neurons in T2DM? Does anti-diabetic treatment have an impact on microglia and/or NPY and POMC neurons? Hereto,
we characterized neurons and microglia in the IFN of T2DM subjects, using post-mortem human brain tissue, with special attention to the possible effects of anti-diabetic treatments.

**Results**

*Distribution of NPY-ir neurons in the IFN is not different between CTRL and T2DM subjects*

The brain size of individual subjects and thus consequently the sagittal length of the hypothalamus varies considerably between subjects. In order to fairly compare neuronal and glial parameters within the IFN and prevent differences between groups of subjects due to differences in brain size, we decided to use the sections around the area in the IFN that showed the highest NPY immunoreactivity (-ir) to study the different neuron and microglial markers. First, sequential sections with 100 sections interval, along the rostro-caudal axis starting from the optic chiasm to the mammillary body of the hypothalamus, were visualized with NPY-ir. NPY-ir neurons are mainly present in the medial and central area of the IFN (Supplemental Figure 1A). Plots of the total number of NPY-ir somas in every 100th section resulted in similar graphs for CTRL (Figure 1A) and T2DM (Figure 1B) subjects. There was no significant difference in area under the curve between T2DM subjects and the matched CTRL subjects (p = 0.96), indicating the shape, size and orientation of the IFN were comparable between the two groups.

![Figure 1. The section selection strategy. (A) Schematic of human hypothalamus showing the areas neuropeptide Y immunoreactivity (NPY-ir) neurons were counted in the infundibular nucleus (IFN) in the hypothalamus along the rostral-caudal axis. (B, C) The distribution pattern of NPY-ir neurons along the rostral to caudal axis in the IFN of the CTRL subjects (B) and the T2DM subjects (C) is similar. 3V, third ventricle; DMH, dorsomedial hypothalamus; fx, fornix; ithp, inferior thalamic peduncle; LV, lateral ventricle; NTL, lateral tuberal nucleus; LHA; lateral hypothalamus; ot, optic tract; PT, paratenial thalamic nucleus; ST, stria terminalis; TM, tuberomammillary hypothalamic nucleus; VMH, ventromedial hypothalamus.](image-url)
Metformin treatment is associated with decreased NPY-ir in the infundibular nucleus, independent of additional insulin treatment.

We further characterized NPY-ir neurons in the central part of the IFN, measuring neuron number, soma size and relative area covered by NPY-ir in both CTRL and T2DM subjects, with T2DM subjects further grouped by the anti-diabetic treatments they had received (Supplemental Figure 2) (Figure 2 A-H). In CTRL subjects, BMI negatively correlated with the number of NPY-ir neurons and with the relative area covered by NPY-ir (Supplemental Figure 3 A1 and C1). Interestingly, in the T2DM subjects, who had matching BMI (Table 1), this correlation was completely absent for all NPY parameters (Supplemental Figure 4 A1, B1 and C1), which might indicate that the possible negative feedback of adiposity on NPY neurons in the CTRL subjects was lost in the T2DM subjects. Regarding the other confounders (HbA1c, post-absorptive glucose, age, post-mortem delay (PMD), fixation time and brain weight), none of these correlated to the NPY parameters in T2DM subjects (Supplemental Figure 4). In CTRL subjects, data on post-absorptive glucose and HbA1c were limited, and therefore we only analyzed the remaining confounders and found no significant correlations (Supplemental Figure 3). Since PMD was not correlated to NPY-ir soma number and size, we concluded that the difference in PMD between CTRL and T2DM groups could not have influenced our results.

We found no differences in NPY neuron number, soma size and relative area of coverage between the T2DM group and the CTRL group (Figure 2 I-K). We then analyzed within the T2DM subjects the effects associated with anti-diabetic treatments. All confounders matched well between treatment groups, except that those who received insulin treatment were younger than those that did not (Table 1). Because metformin and insulin are both known to have an inhibitory effect on appetite and hyperglycemia (Schwartz, Sipols et al. 1992, Paolisso, Amato et al. 1998), we wanted to know if T2DM subjects had different levels of NPY expression based on their treatment. Indeed, we found metformin-associated effects on NPY expression: less neurons, smaller soma and less relative area covered by NPY-ir (Figure 2 L-N, p = 0.001, p = 0.036 and p = 0.0007, respectively). We did not observe an insulin associated effect on any of these NPY-ir parameters (Figure 2 L-N). The observed metformin-associated decrease of NPY-ir parameters indicates a general loss of neuronal function, and may indicate the mechanism through which metformin can reduce appetite, i.e. by regulating NPY activity.
Table 1. Subject group characteristics

<table>
<thead>
<tr>
<th></th>
<th>CTRL</th>
<th>T2DM</th>
<th>p-value</th>
<th>T2DM</th>
<th>T2DM</th>
<th>p-value</th>
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<th>T2DM</th>
<th>p-value</th>
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<tr>
<td>gender</td>
<td>8f / 4m</td>
<td>4f / 16m</td>
<td>0.234</td>
<td>10f / 11m</td>
<td>5f / 6m</td>
<td>0.907</td>
<td>8f / 110m</td>
<td>7f / 7m</td>
<td>0.755</td>
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<tr>
<td>age (years)</td>
<td>78 ± 11.3</td>
<td>80 ± 9.8</td>
<td>0.726</td>
<td>80 ± 9.7</td>
<td>81 ± 10.3</td>
<td>0.686</td>
<td>84 ± 8.2</td>
<td>76 ± 9.7</td>
<td>0.012</td>
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<tr>
<td>PMD (hours)</td>
<td>10.4 ± 6.1</td>
<td>7.2 ± 5.1</td>
<td>0.033</td>
<td>8.2 ± 6.0</td>
<td>5.3 ± 1.5</td>
<td>0.211</td>
<td>7.0 ± 5.1</td>
<td>7.4 ± 5.1</td>
<td>0.352</td>
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<tr>
<td>BMI (kg/m2)</td>
<td>30 ± 4.6</td>
<td>26 ± 4.7</td>
<td>0.054</td>
<td>28 ± 2.7</td>
<td>25 ± 6.1</td>
<td>0.413</td>
<td>25 ± 6.6</td>
<td>28 ± 3.3</td>
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<td>post absorptive glucose</td>
<td>5.6 ± 2.1</td>
<td>7.2 ± 2.3</td>
<td>0.039</td>
<td>7.3 ± 3.0</td>
<td>7.2 ± 1.7</td>
<td>0.949</td>
<td>6.5 ± 1.0</td>
<td>7.7 ± 2.7</td>
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<td>HbA1c (mmol/mol)</td>
<td>36 ± 6.1</td>
<td>63 ± 26.6</td>
<td>0.007</td>
<td>72 ± 33.6</td>
<td>54 ± 16.3</td>
<td>0.341</td>
<td>60 ± 17.0</td>
<td>68 ± 39.8</td>
<td>0.81</td>
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<td>brain weight (gram)</td>
<td>1206 ± 142</td>
<td>1167 ± 123</td>
<td>0.651</td>
<td>1179 ± 134</td>
<td>1144 ± 98</td>
<td>0.46</td>
<td>1158 ± 104</td>
<td>1178 ± 147</td>
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<td>-</td>
<td>ns</td>
<td>-</td>
<td>-</td>
<td>ns</td>
<td>-</td>
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<tr>
<td>month of death</td>
<td>-</td>
<td>-</td>
<td>ns</td>
<td>-</td>
<td>-</td>
<td>ns</td>
<td>-</td>
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<tr>
<td>fixation time (days)</td>
<td>48 ± 15</td>
<td>48 ± 16</td>
<td>0.88</td>
<td>46 ± 17</td>
<td>53 ± 14</td>
<td>0.111</td>
<td>49 ± 17</td>
<td>47 ± 15</td>
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<td>Braak stage</td>
<td>1.6 ± 0.2</td>
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<td>0.146</td>
<td>2.1 ± 1.1</td>
<td>2.1 ± 1.4</td>
<td>0.938</td>
<td>2.4 ± 1.1</td>
<td>1.7 ± 1.1</td>
<td>0.118</td>
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Table 1. Subjects group characteristics. Normal distribution was calculated using D’Agostino & Pearson normality test. Normal distributed data was further analyzed using a student t-test. Data not following a normal distribution were further analyzed using a Mann-Whitney test. Gender differences were calculated using Chi-square test. Time of death and month of death were analyzed using Mardia-Watson-Wheeler test.
Figure 2. Metformin treatment in T2DM subjects is associated with decreased NPY-ir in the IFN, independent of additional insulin treatment. (A - K) Neuropeptide Y immunoreactivity (NPY-ir) neurons in the infundibular nucleus (IFN) of a CTRL subject (A), a T2DM subject with no insulin no metformin (B), with metformin and insulin (C) or with insulin (D) (higher magnification of the areas framed in A - D are shown in E - H respectively). (I - K) Comparison between CTRL and T2DM subjects on: NPY-ir neuronal number (I), NPY-ir soma size (J) and the relative area covered by NPY-ir (K). (L - N) The anti-diabetic treatments-associated effect on NPY-ir neuronal number (L), NPY-ir soma size (M) and the relative area covered by NPY-ir (N). 3V: third ventricle. Scale bars are 500 µm in D, and 50 µm in H. Data represented as mean ± SEM. Significance calculated using a 2-way ANOVA in L-N. All p-values were corrected for multiple testing using the Benjamini-Hochberg criterion, * P < 0.05; *** P < 0.001.
Decreased POMC-ir in the infundibular nucleus in T2DM subjects can be prevented by insulin treatment.

The decrease of NPY-ir raised the question as to whether there are also changes in the other major neuronal population in the IFN, the POMC neurons. Staining of POMC in the central part of the IFN revealed that the POMC region largely overlapped with the NPY region, but extended into a wider more lateral and dorsal area (Supplemental Figure 1B). The staining was predominantly present in the cytoplasm of the cell bodies and less in fibers. Again, we checked for confounders. We found that in CTRL subjects, age negatively correlated with POMC-ir soma size and relative area covered by POMC-ir (Supplemental Figure 5 B2 and C2), indicating a loss of POMC-ir cells due to the ageing process in general. Nevertheless, since we matched the groups for age (Table 1), these correlations will not have influenced our conclusions on group effects. In T2DM subjects, age did not correlate to any POMC parameter (Supplemental Figure 6 A4, B4 and C4). Moreover, the other confounders did not correlate to any of the POMC-ir parameters in the CTRL subjects (Supplemental Figure 5), nor in T2DM subjects (Supplemental Figure 6).

We found that, compared to the matched CTRL group, the POMC-ir neurons and the area covered by POMC-ir were significantly lower in the T2DM group (Figure 3I and K, q = 0.04 and q = 0.0499, respectively), indicating a general decrease of POMC expression in T2DM subjects, although no difference in POMC-ir soma size was found (Figure 3J). Moreover, these data also suggest that the T2DM pathogenesis has a distinctive detrimental impact on the cell biology of NPY and POMC neurons.

As mentioned before, the only confounding factor between treatment groups was age, e.g. insulin treated subjects died at significantly younger age than T2DM subjects without insulin treatment (Table 1, p = 0.01), but this did not influence our conclusions as in the T2DM group age showed no confounding effect on the POMC-ir parameters (Supplemental Figure 6). The remaining confounders matched well between the T2DM groups (Table 1). We found contrasting effects associated to the anti-diabetic treatments on POMC-ir neurons. In those treated with metformin, a combination with insulin treatment is associated with larger POMC-ir somas (Fig 3M, p = 0.003), whereas metformin treatment associated with smaller POMC-ir somas (Fig 3M, p = 0.031). T2DM subjects in general have decreased levels of POMC expression, which can be prevented by long-term insulin treatment. These results imply a diverse impact of insulin and metformin on NPY and POMC expression.
Figure 3. Decreased POMC-ir in the IFN of T2DM subjects can be prevented by insulin treatment. (A - H) Pro-opiomelanocortin immunoreactive (POMC-ir) neurons in the infundibular nucleus (IFN) of a CTRL subject (A), T2DM subject with no insulin no metformin (B), with metformin (C) or with insulin (D) (higher magnification of the areas framed in A - D are shown in E - H respectively). (I - K) Comparison between CTRL and T2DM of: POMC-ir neuronal number (I), POMC-ir soma size (J) and the relative area covered by POMC-ir (K). (L - N) The anti-diabetic treatments-associated effect on POMC-ir neuronal number (L), POMC-ir soma size (M) and the relative area covered by POMC-ir (N). 3V: third ventricle. Scale bars are 500 µm in D, and 50 µm in H. Data represented as mean ± SEM. Significance calculated using an ordinary t-test in I, a Mann-Whitney test in K and a 2-way ANOVA in M. All p-values were corrected for multiple testing using the Benjamini-Hochberg criterion, * P < 0.05; ** P < 0.01.
Figure 4. T2DM subjects treated with metformin show a regional specific decrease in the number of iba1-ir microglial soma and ramifications in the IFN. (A - H) Ionized calcium-binding adapter molecule 1 immunoreactive (iba1-ir) microglia in the infundibular nucleus (IFN) of a CTRL subject (A), T2DM subject with no insulin no metformin (B), with metformin and insulin (C) or with insulin (D) (higher magnification of the areas framed in A - D are shown in E - H respectively). (I - K) Comparison in the neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) region of: iba1-ir soma number (I), iba1-ir soma size (J) and the number of iba1-ir ramifications (K). (L - N) The anti-diabetic treatments associated effects in the NPY and POMC region on iba1-ir soma number (L), iba1-ir soma size (M) and the number of iba1-ir ramifications (N). 3V: third ventricle. Scale bars are 500 µm in D, and 50 µm in H. Data represented as mean ± SEM. Significance calculated using a 2-way ANOVA in L and N. All p-values were corrected for multiple testing using the Benjamini-Hochberg criterion, * P < 0.05.
T2DM subjects treated with metformin show a regional specific decrease in the number of iba1-ir microglial soma and ramifications.

To investigate the microglial changes in the IFN of T2DM subjects, we profiled the microglia using ionized calcium-binding adapter molecule 1 (iba1), as it is the best structural marker for visualizing microglia in grey matter (Hendrickx, van Eden et al. 2017). The iba1-ir staining showed microglial cells throughout the IFN. The iba1 staining was present in the cell bodies as well as in the ramifications. Since due to the limited thickness of the sections, the full morphology of individual iba1-ir cell was impossible to determine, we quantified iba1-ir soma number and size as well as the number of ramifications. An increase of these parameters compared to normal microglia is an indication of higher microglial activity.

We investigated the same confounding factors as before and found that, in the NPY region of CTRL subjects, brain weight positively correlated to the number of iba1-ir soma and ramifications (Supplemental Figure 7 A3 and C3). A previous study reported a smaller brain weight in association with a loss of myelination due to lacking insulin-like growth factor 1 (IGF1) in CD11c (mainly expressed by macrophages and to less extend by microglia) positive cells (Wlodarczyk, Holtman et al. 2017), whether IGF1 in aged human brain microglia is also involved in maintaining myelination needs to be further studied. We also found that BMI positively correlated to iba1-ir soma size (Supplemental Figure 7 B1), pointing to a possible association between obesity and microglial activity. Nevertheless, our analysis on group differences will not have been influenced by these two confounding factors, because we matched all groups for both BMI and brain weight (Table 1). In the POMC region of CTRL subjects and T2DM subjects, we found no correlations between iba1-ir and brain weight, BMI, post-absorptive glucose and HbA1c (Supplemental Figure 8 and 10).

Because diet-induced obesity and pre-diabetes as well as hypothalamic neuronal dysfunction are associated to increased microglial activity in animal studies, we expected an increase of microglia in our T2DM subjects as well. However, we did not find any differences in iba1-ir parameters between T2DM and the CTRL subjects, not in the NPY region nor in the POMC region (Figure 4 I-K).

With the anti-inflammatory properties of both metformin and insulin in mind, we anticipated a treatment associated effect on microglial activity in these T2DM subjects. Surprisingly, we only found an effect associated to metformin treatment; 2-way ANOVA showed that metformin treatment associated with less iba1-ir cell bodies and less iba1-ir ramifications (Figure 4L and N, p = 0.037, p = 0.012, respectively). We found no insulin associated effect on any of the iba1 parameters (Figure 4L – N).
Locally decreased TMEM119-ir in the NPY area in the IFN of the T2DM subjects treated with metformin.

In order to gain more insight in the activation state of the microglia, we also profiled transmembrane protein 119 immunoreactivity (TMEM119-ir). This trans-membrane protein is known to negatively correlate to microglia activity and is mainly expressed by residential microglia (Zrzavy, Hametner et al. 2017, van der Poel, Ulas et al. 2019). We saw that TMEM119 showed a similar staining pattern as iba1, both present on the cell bodies as on the ramifications (Figure 5 A-D). Likewise, the full morphology of each microglia was impossible to determine
because of the limited thickness of the sections.
In CTRL subjects, BMI had a great impact on TMEM119-ir microglia, as it negatively correlated to the number of ramifications in both the NPY and POMC region (Supplemental Figure 11 C1 and 12 C1) and negatively correlated to the number of somas in the POMC region (Supplemental Figure 12 A1), again pointing to an association between obesity and microglial activity. Contrary to the iba1-ir results, the TMEM119-ir parameters did not correlate to brain weight (Supplemental Figure 11 and 12, A3, B3, C3), which suggests that only infiltrating macrophages might be linked to brain atrophy, which seems consistent to the aforementioned association between brain weight and the lack of IGF1 in CD11c macrophages/microglia (Wlodarczyk, Holtman et al. 2017). In T2DM subjects, we found no further significant confounding in either the NPY or the POMC region (Supplemental Figure 13 and 14).

The average number of TMEM119-ir microglia was roughly half of the number of iba1-ir microglia (Figure 4 and 5). Similar to the iba1-ir results, there were no differences in TMEM119-ir microglia between T2DM and CTRL subjects (Figure 5 I-K). We also did not find any treatment associated effects in the POMC region (Figure 5 L-N). In the NPY region, however, there was a metformin associated decrease of the number of TMEM119-ir ramifications (Figure 5 N, p = 0.011). In addition, the combined effect of insulin plus metformin treatment seemed to increase TMEM119-ir microglial soma size in the NPY region (Figure 5M, p = 0.011).

The relation between iba1-ir and TMEM119-ir cells may shed some light on the regulation of resident microglia (expressing TMEM119-ir and iba1-ir) and infiltrating macrophages (expressing iba1-ir, but less TMEM119-ir) in the IFN. We therefore looked into the correlation between these two markers. Intriguingly, in the NPY region of CTRL subjects, we found no correlation between the number of iba1 and TMEM119 expressing cells (Figure 6A). Which suggests either an increased activity of microglia or an increase of non-residential microglia, likely invading macrophages. In contrast, in the NPY region of T2DM subjects, iba1-ir and TMEM119-ir showed a significant correlation (Figure 6B), indicating that changes in the number of total immune cells are partly the result of a changed number of residential microglia. In the POMC region, we found no correlations between the number of iba1-ir cells and TMEM119-ir cells, in either the CTRL or T2DM subjects (Figure 6C and D), again, suggesting likely a higher presence of invaded macrophages in this region, compared to the NPY region of T2DM subjects.

**Number of microglia are positively correlated to number of NPY-ir neurons**

The local decrease of microglia in the NPY region raised the question whether this correlated to the number of neurons present in this region. We therefore investigated if there was a correlation between the number of neurons and microglia in the two different regions.

In CTRL subjects, the number of NPY-ir neurons did not correlate to the number of iba1-ir cells in that region (Figure 6E). On the other hand, in T2DM subjects, the number of NPY-ir neurons...
neurons positively correlated to the number of iba1-ir cells (Figure 6F, p = 0.003). Regarding the correlations between NPY-ir neurons and TMEM119 microglia, we found no correlation in CTRL (Figure 6G), but also a positive correlation in T2DM subjects (Figure 6H, p = 0.043). Contrary to the NPY-ir neurons, the number of POMC-ir neurons did not correlate with the number of iba1-ir or TMEM119-ir microglia in either CTRL or T2DM subjects (Figure 6 I-L). Together these results suggest that in T2DM, there are more interactions between NPY neurons and microglia/macrophages, whereas the lack of interaction between POMC-ir neurons and microglia is likely responsible for the loss of POMC-ir neurons in the T2DM.

Figure 6. The association between the iba1-ir microglia and the TMEM119-ir microglia and the association between the number of neurons and the number of microglia in the infundibular nucleus. (A, B) Plots of the number of Ionized calcium-binding adapter molecule 1 immunoreactive (iba1-ir) microglia with the number of transmembrane protein 119 immunoreactive (TMEM119-ir) microglia in the neuropeptide Y (NPY) region of CTRL subjects (A) and T2DM subjects (B). (C, D) Plots of the number of iba1-ir microglia with the number of TMEM119-ir microglia in the pro-opiomelanocortin (POMC) region of CTRL subjects (C) and T2DM subjects (D). (E - H) The number of NPY-ir neurons plotted against the number of iba1-ir microglia in CTRL subjects (E) and T2DM subjects (F), and against the number of TMEM119-ir microglia in CTRL subjects (G) and T2DM subjects (H). (I - L) The number of POMC-ir neurons plotted against the number of iba1-ir microglia in CTRL subjects (I) and T2DM subjects (J), and against the number of TMEM119-ir microglia in CTRL subjects (K) and T2DM subjects (L).
Discussion

To our best knowledge, this is the first study to examine the relationship between different anti-diabetic treatments of T2DM patients and alterations in neurons and microglia in the human hypothalamus. We found clear differences in POMC-ir, NPY-ir, iba1-ir and TMEM119-ir cell numbers and cell sizes in T2DM subjects, especially when taking into account their anti-diabetic treatments. POMC-ir was decreased in the T2DM group as a whole, but this decrease was not observed in subjects that had received insulin treatment. In contrast, NPY-ir and iba1-ir/TMEM119-ir in T2DM subjects were not different compared to the CTRL subjects. However, within the T2DM subjects, NPY-ir was significantly lower in T2DM subjects treated with metformin. Moreover, iba1-ir and TMEM119-ir were also decreased in T2DM subjects that had received metformin treatment, exclusively in the NPY region. Finally, T2DM individuals without metformin treatment had larger NPY-ir neuronal soma than CTRL subjects had.

The role of the hypothalamic cells in the pathophysiology of T2DM has been studied extensively in animal models. However, studies in the hypothalamus of human T2DM patients are scarce and the few studies performed so far show conflicting results. One study showed an increase of NPY-ir neurons without changes in the POMC-ir neurons (Saderi, Salgado-Delgado et al. 2012), whereas another study showed no effect on NPY-ir neurons, but a decrease of POMC-ir neurons (Alkemade, Yi et al. 2012). Both studies used post-mortem brain tissue and matched the T2DM and CTRL subjects for sex, age and PMD. As only one study reported the BMI of the subjects this might explain the conflicting results, although the same study showed there is no correlation between BMI and number of NPY-ir or POMC-ir neurons (Alkemade, Yi et al. 2012). In addition to possible (but unascertainable) differences in diet composition and genetic background, the one clear and major difference between these two studies is the anti-diabetic treatment the subjects received. The T2DM subjects from Saderi et al. received “very limited to nil” treatment (Saderi, Salgado-Delgado et al. 2012), whereas those from Alkemade et al. consisted only of T2DM subjects “requiring treatment with oral antidiabetics or insulin” (Alkemade, Yi et al. 2012). Thus, comparison of T2DM subjects with a different treatment history can lead to different results. The anti-diabetic treatment itself might be accountable, or it might be because these patients are in different stages of the disease, requiring different treatments.

Metformin is widely used as the standard first line agent to treat type 2 diabetes. Consequently, patients only on metformin treatment are in a different disease stage than those requiring insulin treatment. Metformin decreases intestinal glucose uptake, decreases hepatic glucose production and increases insulin sensitivity (Takahara, Kaneto et al. 2012, Carvajal, Rosas et al. 2013, Kristensen, Treebak et al. 2014, Duca, Cote et al. 2015). Furthermore, metformin inhibits food intake and reduces body weight gain in animals (Chau-Van, Gamba et al. 2007, Hou, Venier et al. 2010, Matsui, Hirasawa et al. 2010). Also in humans metformin is known to decrease food consumption, body weight and appetite (Lee and Morley 1998, Paolisso, Amato...
et al. 1998, Schultes, Oltmanns et al. 2003). The exact mechanisms underlying these effects are unclear, but it seems likely that the hypothalamus as the control center of energy homeostasis is involved. Some studies have explored the relation between metformin treatment and POMC and NPY expression. In diabetic rats, metformin inhibited the hypothalamic expression of NPY mRNA, but had no effect on POMC mRNA expression (Chau-Van, Gamba et al. 2007). In our study, we found a clear association of metformin treatment with a decrease of all NPY-ir parameters, indicating that in T2DM patients, long-term metformin treatment might be able to suppress NPY expression and its orexigenic effect. The lack of a correlation between NPY-ir and glycemic markers indicates that metformin does not affect NPY-ir through its glucose-lowering properties, but rather by a direct effect on NPY-ir neurons.

Previous animal studies show that hypothalamic inflammation is involved in disrupting the hypothalamic control of energy homeostasis, partly reflected by an increase of microglia number and size (Thaler, Yi et al. 2012). The effect of metformin on microglial function is relatively unknown, but results from animal studies generally show that metformin reduces microglial expression of pro-inflammatory cytokines and that this has a neuroprotective effect (Zhu, Jiang et al. 2015, Oliveira, Nunes et al. 2016, Pan, Sun et al. 2016, Tao, Li et al. 2018). Our study did not reveal differences in microglial markers between T2DM and CTRL subjects in general, but T2DM subjects with metformin treatment did have less microglial cell bodies and ramifications in the NPY region compared to T2DM subjects without metformin treatment. This is opposite to the increase of microglia seen in the animal studies mentioned above. This discrepancy could be explained by the fact that these animals usually do not get anti-diabetic treatment as humans do. Another explanation could be that in animal studies the animals stay in a pre-diabetic stage and haven’t reached the actual diabetic phase, which would mean we are looking at different phases of the disease. Additionally, the microglia activation found in animals is already present within weeks after the start of the intervention (Thaler, Yi et al. 2012), whereas our patient cohort consisted of relatively aged subjects, indicating our results reflect long-term effects of diabetes on microglia.

Interestingly, besides the positive correlations between the number of NPY-ir neurons and the number of iba1-ir / TMEM119-ir microglia in T2DM subjects, we also observed that the metformin-treated group had the lowest number of NPY-ir neurons. Thus, it seems unlikely that inhibition of microglia activity has a neuroprotective effect. Rather, it indicates that microglia activity might adapt to the neuronal demand for scavenging activity, i.e., less demand from neurons results in less microglial scavenging activity. Such a correlation was only found between microglia and NPY neurons, suggesting that interaction between microglia and NPY neurons is substantially different from the interaction between microglia and the POMC neurons in T2DM. In our previous animal study, we found that on a high fat diet reactive microglia moved closer to POMC neurons (Yi, Walter et al. 2017). This raises the possibility that in a metabolic disordered brain, POMC neurons are more vulnerable and have a higher demand
for scavenging activity by microglia. However, in the long run, this microglia - POMC neuron interaction becomes defective or even detrimental and that results in POMC neuronal loss, as we observed in the current study. In contrast, NPY neurons might possess a stronger or more adequate survival mechanism. Different neuropeptides and neurotransmitters produced by NPY and POMC neurons might be underlying these specific neuron-microglial interactions. Indeed, microglia express receptors for many neurotransmitters, including NPY and α-MSH (Lindberg, Hjorth et al. 2005, Ferreira, Santos et al. 2011). Moreover, chemical species like reactive oxygen species (ROS) are known to play important roles in the central regulation of energy metabolism that might differ between NPY and POMC neurons (Diano, Liu et al. 2011) and therefore result in a differential role of microglia in the vicinity of these neurons.

In conclusion, our study shows that T2DM pathology differently affects the expression of the neuropeptides POMC and NPY in the hypothalamus in relation to the anti-diabetic treatment. Microglia are not significantly affected by T2DM pathology itself, but rather by the anti-diabetic treatments.

Methods

Subjects information

All brain materials were obtained from the Netherlands Brain Bank. The donor or their next of kin gave informed consent for a brain autopsy and for the use of the brain material and medical records for research purposes. Subjects who died with severe inflammation associated with brain tumor, encephalitis or sepsis were excluded, because of known confounding (Roos 2014, Quail and Joyce 2017, Zrzavy, Höftberger et al. 2019). Subjects diagnosed with an eating disorder or type 1 diabetes were also excluded. Subjects that had Braak stage V-VI (Braak, Alafuzoff et al. 2006) were excluded, as indications of severe dementia, although Alzheimer neuropathology is minimal in the IFN (van de Nes, Kamphorst et al. 1998). When medical records did not report severe dementia, subjects lacking a Braak stage analysis were included as non-demented CTRL (Supplemental Table 1).

In total, 49 post-mortem human hypothalamic samples were studied, 32 subjects diagnosed with T2DM and 17 non-diabetic matched CTRL subjects. Among these 32 T2DM subjects, 13 subjects received neither metformin nor insulin treatment (no anti-diabetic medications (n = 8) or other oral anti-diabetic medications (n=5)), five subjects received metformin but no insulin treatment prior to their death, eight subjects were under insulin treatment without additional metformin treatment and six subjects received both metformin and insulin treatment (supplemental Figure 2). The duration of T2DM could not be determined for each individual, due to the fact that the start of diabetic pathology could be far earlier than the diagnosis and start of treatment.

T2DM and CTRL groups matched for sex, age, BMI, brain weight, time of death, month of death, fixation time and Braak stage, but differed in PMD (Table 1). Data on the latest post-
absorptive blood glucose and HbA1c, a measure of 3-month average glucose levels, although incomplete, are also presented in Table 1. The T2DM subgroups, when separated according to metformin or insulin treatment, matched for all the previously mentioned confounders (Table 1). An overview of more details of each individual subject, medication use, clinical diagnosis and cause of death is provided in Supplemental Table 1.

**Histology**

After autopsy, the isolated hypothalamic tissues were immediately immersed in formalin and fixed at room temperature for one to two months (Supplemental Table 1). Tissues were then ethanol-dehydrated, toluene-cleared and paraffin-embedded. Six μm sequential hypothalamic sections were sectioned along the rostro-caudal axis from the lamina terminalis to the mammillary bodies. The anatomical orientation and rostro-caudal range of the IFN was first determined by Nissl staining, and subsequently precisely established by NPY-ir of every 100th section (Figure 1). For every subject the section with the highest number of NPY-ir soma was determined and all the following staining’s were performed on 2 sections in close proximity to this section. For both procedures, sections were mounted on glass slides (superfrost+) and dried on a 37°C heating plate. After 48 hours, sections were deparaffinized in 100% xylene, rehydrated in grading ethanol (100% - 50%) and rinsed in distilled water. The immunohistochemical procedure of the NPY staining is similar to that of the other staining’s and is described below.

**Immunohistochemistry**

Heat-induced epitope retrieval was performed in pH 6.0 citrate buffer using microwave treatment (10 min at 700 W) before incubation with POMC, Iba1 and TMEM119 (NPY does not require antigen retrieval). After cooling, sections were treated with 3% H₂O₂ in Tris-buffered saline (TBS; 0.05 M Tris, 0.15 M NaCl, pH 7.6) for 10 minutes, washed in TBS, and incubated with the primary antibody overnight at 4°C. The primary antibodies against POMC and NPY were diluted in SUMI-milk (5% nonfat milk, 0.25% gelatin, 0.5% Triton X-100 in TBS) and those against Iba1 and TMEM119, i.e., two markers for microglia, were diluted in SUMI without milk. The next day, sections were washed in TBS, incubated for 60 minutes with biotinylated horse anti-rabbit IgG antibody (1:400, Vector Laboratories), washed in TBS, incubated with avidin–biotin complex (1:800, Vectastain Elite ABC kit; Vector Laboratories Inc.) and washed in TBS. Finally, sections were incubated in 0.5 mg/ml 3,3’-Diaminobenzidine (Sigma Chemical Co., St. Louis, MO) in TBS containing 0.2% ammonium nickel sulfate (DAB/Ni) (BDH; Brunschwig, Amsterdam, The Netherlands) and 0.01% H₂O₂ (Merck, Darmstadt, Germany) for 20 min. The reaction was stopped in distilled water. Afterwards, sections were ethanol dehydrated, xylene cleared and Entelan cover slipped. Primary antibodies used were: rabbit polyclonal anti-POMC (1:1000, Phoenix Pharmaceuticals, 27-52), rabbit polyclonal anti-NPY (1:1000, Netherlands Institute for Brain Research; Niepke 261188) (Fliers, Unmehopa et al.
rabbit polyclonal anti-iba1 (1:200, Synaptic Systems, 234003) and rabbit monoclonal anti-TMEM119 (1:400, Abcam, ab209064). The specificities of these primary antibodies were confirmed by comparison with negative control staining.

**Quantitative analysis**

Quantitative image analysis was performed by a researcher blind to the clinical information of the subjects. Neuronal and microglial cell numbers were counted using Fiji, an ImageJ distribution (Schindelin, Arganda-Carreras et al. 2012). Quantification of POMC and NPY expressing neurons was performed by manually outlining the area of interest based on the location of positive signal. Subsequently, using the “particle analysis” tool, the number and size of all immunoreactive positive particles in the outline were calculated, particles larger than 30 µm² were considered a neuronal soma. This size was determined in a pilot study for both NPY-ir and POMC-ir neurons, measuring the average size of the smallest nucleolus-containing neurons. Total soma number was divided by the area of the outline, resulting in soma number / mm². The size of all somata was averaged and resulted in an average soma size (µm²). Finally, the percentage of outlined area occupied by total immunoreactive positive particles was calculated as % area masked.

For microglia visualized by iba1-ir and TMEM119-ir, the number of positive cells was calculated in the outlines generated by the corresponding POMC and NPY staining’s (Supplemental Figure 1C). Positive particles larger than 20 µm² and smaller than 100 µm² (size determined in pilot study) were considered a microglial soma, resulting in soma number / mm². Soma size of all somas was averaged, resulting in average soma size (µm²). The number of particles between 6 µm² and 20 µm² were counted as number of ramifications. Total number of ramifications was divided by area of outline, resulting in ramification number / mm².

**Statistics**

We performed a D’Agostino & Pearson normality test to determine if data were normally distributed. Normal distributed data were further analyzed using a 2-tailed student’s t-test. Data not following a normal distribution were further analyzed using a Mann-Whitney test. We controlled for multiple testing using the Benjamini-Hochberg criterion (Benjamini and Hochberg 1995), resulting in an adjusted p-value (q-value). Treatment associated effects were analyzed using a 2-way ANOVA. Correlations were measured using linear regression. A p-value smaller than 0.05 was considered statistically significant. All statistical tests were performed using GraphPad Prism 8.12.
Study approval

All brain material was obtained from the Netherlands Brain Bank. The donor or their next of kin gave informed consent for a brain autopsy and for the use of the brain material and medical records for research purposes.

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References


Supplemental figures

**Supplemental Figure 1. Outlines of immunoreactivity of neurons and microglia in the IFN.** (A) The neuropeptide-Y immunoreactive (NPY-ir) area is framed by a blue line in the infundibular nucleus (IFN) for data analysis; (B) The pro-opiomelanocortin immunoreactive (POMC-ir) area is framed by a red line in the IFN for data analysis; (C) The ionized calcium-binding adapter molecule 1 immunoreactive (Iba1-ir) microglia in the IFN were separately analyzed in both the areas framed by the blue and the red lines; (D) The transmembrane protein 119 immunoreactive (TMEM119-ir) microglia were separately analyzed in both the areas framed by the blue and the red lines. Panel A is cropped from Figure 2A, panel B from Figure 3A, panel C from Figure 4A and panel D from Figure 5A. 3V: third ventricle. Scale bar: 500 µm in D.

**Supplemental Figure 2. An overview of the treatment history of the T2DM subjects which received either metformin and/or insulin treatment.** Metformin is shown in pink, insulin in orange and the combination is shown in olive green.
Supplemental Figure 3. Confounders analysis with NPY-ir in CTRL subjects. (A) Plots of the number of neuropeptide-Y immunoreactive (NPY-ir) neurons in CTRL subjects according to body mass index (BMI) (A1), age (A2), brain weight (A3), post-mortem delay (PMD) (A4) and fixation time (A5). (B) Plots of average soma size of NPY-ir neurons in CTRL subjects according to BMI (B1), age (B2), brain weight (B3), PMD (B4) and fixation time (B5). (C) Plots of relative area covered by NPY-ir neurons in CTRL subjects according to BMI (C1), age (C2), brain weight (C3), PMD (C4) and fixation time (C5).
Supplemental Figure 4. Confounders analysis with NPY-ir in T2DM subjects. (A) Plots of the number of neuropeptide-Y immunoreactive (NPY-ir) neurons in T2DM subjects according to body mass index (BMI) (A1), post absorptive glucose (A2), HbA1c (A3), age (A4), brain weight (A5), post-mortem delay (PMD) (A6) and fixation time (A7). (B) Plots of average soma size of NPY-ir neurons in T2DM subjects according to BMI (B1), post absorptive glucose (B2), HbA1c (B3), age (B4), brain weight (B5), PMD (B6) and fixation time (B7). (C) Plots of relative area covered by NPY-ir neurons in T2DM subjects according to BMI (C1), post absorptive glucose (C2), HbA1c (C3), age (C4), brain weight (C5), PMD (C6) and fixation time (C7).
Supplemental Figure 5. Confounders analysis with POMC-ir in CTRL subjects. (A) Plots of the number of pro-opiomelanocortin immunoreactive (POMC-ir) neurons in CTRL subjects according to body mass index (BMI) (A1), age (A2), brain weight (A3), post-mortem delay (PMD) (A4) and fixation time (A5). (B) Plots of average soma size of POMC-ir neurons in CTRL subjects according to BMI (B1), age (B2), brain weight (B3), PMD (B4) and fixation time (B5). (C) Plots of relative area covered by POMC-ir neurons in CTRL subjects according to BMI (C1), age (C2), brain weight (C3), PMD (C4) and fixation time (C5).
Supplemental Figure 6. Confounders analysis with POMC-ir in T2DM subjects. (A) Plots of the number of pro-opiomelanocortin immunoreactive (POMC-ir) neurons in T2DM subjects according to body mass index (BMI) (A1), post absorptive glucose (A2), HbA1c (A3), age (A4), brain weight (A5), post-mortem delay (PMD) (A6) and fixation time (A7). (B) Plots of average soma size of POMC-ir neurons in T2DM subjects according to BMI (B1), post absorptive glucose (B2), HbA1c (B3), age (B4), brain weight (B5), PMD (B6) and fixation time (B7). (C) Plots of relative area covered by POMC-ir neurons in T2DM subjects according to BMI (C1), post absorptive glucose (C2), HbA1c (C3), age (C4), brain weight (C5), PMD (C6) and fixation time (C7).
Supplemental Figure 7. Confounders analysis with iba1-ir in NPY region of CTRL subjects. 
(A) Plots of the number of ionized calcium-binding adapter molecule 1 immunoreactive (iba1-ir) microglia in the neuropeptide Y (NPY) region of CTRL subjects according to body mass index (BMI) (A1), age (A2), brain weight (A3), post-mortem delay (PMD) (A4) and fixation time (A5). (B) Plots of average soma size of iba1-ir microglia in the NPY region of CTRL subjects according to BMI (B1), age (B2), brain weight (B3), PMD (B4) and fixation time (B5). (C) Plots of the number of iba1-ir microglial ramifications in the NPY region of CTRL subjects according to BMI (C1), age (C2), brain weight (C3), PMD (C4) and fixation time (C5).
Supplemental Figure 8. Confounders analysis with iba1-ir in POMC region of CTRL subjects.

(A) Plots of the number of ionized calcium-binding adapter molecule 1 immunoreactive (iba1-ir) microglia in the pro-opiomelanocortin (POMC) region of CTRL subjects according to body mass index (BMI) (A1), age (A2), brain weight (A3), post-mortem delay (PMD) (A4) and fixation time (A5).

(B) Plots of average soma size of iba1-ir microglia in the pro-opiomelanocortin (POMC) region of CTRL subjects according to BMI (B1), age (B2), brain weight (B3), PMD (B4) and fixation time (B5).

(C) Plots of the number of iba1-ir microglial ramifications in the POMC region of CTRL subjects according to BMI (C1), age (C2), brain weight (C3), PMD (C4) and fixation time (C5).
Supplemental Figure 9. Confounders analysis with iba1-ir in NPY region of T2DM subjects.
(A) Plots of the number of ionized calcium-binding adapter molecule 1 immunoreactive (iba1-ir) microglia in the neuropeptide Y (NPY) region of T2DM subjects according to body mass index (BMI) (A1), post absorptive glucose (A2), HbA1c (A3), age (A4), brain weight (A5), post-mortem delay (PMD) (A6) and fixation time (A7).

(B) Plots of average soma size of iba1-ir microglia in the NPY region of T2DM subjects according to BMI (B1), post absorptive glucose (B2), HbA1c (B3), age (B4), brain weight (B5), PMD (B6) and fixation time (B7).

(C) Plots of iba1-ir microglial ramifications in the NPY region of T2DM subjects according to BMI (C1), post absorptive glucose (C2), HbA1c (C3), age (C4), brain weight (C5), PMD (C6) and fixation time (C7).
Supplemental Figure 10. Confounders analysis with iba1-ir in POMC region of T2DM subjects. (A) Plots of the number of ionized calcium-binding adapter molecule 1 immunoreactive (iba1-ir) microglia in the pro-opiomelanocortin (POMC) region of T2DM subjects according to body mass index (BMI) (A1), post absorptive glucose (A2), HbA1c (A3), age (A4), brain weight (A5), post-mortem delay (PMD) (A6) and fixation time (A7). (B) Plots of average soma size of iba1-ir microglia in the POMC region of T2DM subjects according to BMI (B1), post absorptive glucose (B2), HbA1c (B3), age (B4), brain weight (B5), PMD (B6) and fixation time (B7). (C) Plots of iba1-ir microglial ramifications in the POMC region of T2DM subjects according to BMI (C1), post absorptive glucose (C2), HbA1c (C3), age (C4), brain weight (C5), PMD (C6) and fixation time (C7).
Supplemental Figure 11. Confounders analysis with TMEM-ir in NPY region of CTRL subjects.

(A) Plots of the number of transmembrane protein 119 immunoreactive (TMEM119-ir) microglia in the neuropeptide Y (NPY) region of CTRL subjects according to body mass index (BMI) (A1), age (A2), brain weight (A3), post-mortem delay (PMD) (A4) and fixation time (A5).

(B) Plots of average soma size of TMEM119-ir microglia in the NPY region of CTRL subjects according to BMI (B1), age (B2), brain weight (B3), PMD (B4) and fixation time (B5).

(C) Plots of the number of TMEM119-ir microglial ramifications in the NPY region of CTRL subjects according to BMI (C1), age (C2), brain weight (C3), PMD (C4) and fixation time (C5).
Supplemental Figure 12. Confounders analysis with TMEM119-ir in POMC region of CTRL subjects. (A) Plots of the number of transmembrane protein 119 immunoreactive (TMEM119-ir) microglia in the pro-opiomelanocortin (POMC) region of CTRL subjects according to body mass index (BMI) (A1), age (A2), brain weight (A3), post-mortem delay (PMD) (A4) and fixation time (A5). (B) Plots of average soma size of TMEM119-ir microglia in the POMC region of CTRL subjects according to BMI (B1), age (B2), brain weight (B3), PMD (B4) and fixation time (B5). (C) Plots of the number of TMEM119-ir microglial ramifications in the POMC region of CTRL subjects according to BMI (C1), age (C2), brain weight (C3), PMD (C4) and fixation time (C5).
Supplemental Figure 13. Confounders analysis with TMEM119-ir microglia in NPY region of T2DM subjects. (A) Plots of the number of transmembrane protein 119 immunoreactive (TMEM119-ir) microglia in the neuropeptide Y (NPY) region of T2DM subjects according to body mass index (BMI) (A1), post absorptive glucose (A2), HbA1c (A3), age (A4), brain weight (A5), post-mortem delay (PMD) (A6) and fixation time (A7). (B) Plots of average soma size of TMEM119-ir microglia in the NPY region of T2DM subjects according to BMI (B1), post absorptive glucose (B2), HbA1c (B3), age (B4), brain weight (B5), PMD (B6) and fixation time (B7). (C) Plots of TMEM119-ir microglial ramifications in the NPY region of T2DM subjects according to BMI (C1), post absorptive glucose (C2), HbA1c (C3), age (C4), brain weight (C5), PMD (C6) and fixation time (C7).
Supplemental Figure 14. Confounders analysis with TMEM119-ir microglia in POMC region of T2DM subjects. (A) Plots of the number of transmembrane protein 119 immunoreactive (TMEM119-ir) microglia in the pro-opiomelanocortin (POMC) region of T2DM subjects according to body mass index (BMI) (A1), post absorptive glucose (A2), HbA1c (A3), age (A4), brain weight (A5), post-mortem delay (PMD) (A6) and fixation time (A7). (B) Plots of average soma size of TMEM119-ir microglia in the POMC region of T2DM subjects according to BMI (B1), post absorptive glucose (B2), HbA1c (B3), age (B4), brain weight (B5), PMD (B6) and fixation time (B7). (C) Plots of TMEM119-ir microglial ramifications in the POMC region of T2DM subjects according to BMI (C1), post absorptive glucose (C2), HbA1c (C3), age (C4), brain weight (C5), PMD (C6) and fixation time (C7).
Chapter 3

Loss of arginine vasopressin- and vasoactive intestinal polypeptide-containing neurons and glial cells in the suprachiasmatic nuclei of individuals with type 2 diabetes


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# Rick Hogenboom and Martin J. Kalsbeek contributed equally to this study.
Abstract

Aims/hypothesis: The central pacemaker of the mammalian biological timing system is located within the suprachiasmatic nucleus (SCN) in the anterior hypothalamus. Together with the peripheral clocks, this central brain clock ensures a timely, up-to-date and proper behaviour for an individual throughout the day–night cycle. A mismatch between the central and peripheral clocks results in a disturbance of daily rhythms in physiology and behaviour. It is known that the number of rhythmically expressed genes is reduced in peripheral tissue of individuals with type 2 diabetes mellitus. However, it is not known whether the central SCN clock is also affected in the pathogenesis of type 2 diabetes. In the current study, we compared the profiles of the SCN neurons and glial cells between type 2 diabetic and control individuals.

Methods: We collected post-mortem hypothalamic tissues from 28 type 2 diabetic individuals and 12 non-diabetic control individuals. We performed immunohistochemical analysis for three SCN neuropeptides, arginine vasopressin (AVP), vasoactive intestinal polypeptide (VIP), and neurotensin (NT), and for two proteins expressed in glial cells, ionised calcium-binding adapter molecule 1 (IBA1, a marker of microglia) and glial fibrillary acidic protein (GFAP, a marker of astroglial cells).

Results: The numbers of AVP immunoreactive (AVP-ir) and VIP-ir neurons and GFAP-ir astroglial cells in the SCN of type 2 diabetic individuals were significantly decreased compared with the numbers in the SCN of the control individuals. In addition, the relative intensity of AVP immunoreactivity was reduced in the individuals with type 2 diabetes. The number of NT-ir neurons and IBA1-ir microglial cells in the SCN was similar in the two groups.

Conclusions/interpretation: Our data show that type 2 diabetes differentially affects the numbers of AVP- and VIP-expressing neurons and GFAP-ir astroglial cells in the SCN, each of which could affect the daily rhythmicity of the SCN biological clock machinery. Therefore, for effectively treating type 2 diabetes, lifestyle changes and/or medication to normalise central biological clock functioning might be helpful.

Keywords: Astroglial cells, Biological clock, Insulin resistance, Microglia, Neurotensin, Rhythmicity, Type 2 diabetes mellitus
Introduction
In mammals, the circadian timing system plays a critical role in coordinating the daily and seasonal rhythmicity of all physiological and behavioural processes in the body. The master pacemaker of this timing system is located in the suprachiasmatic nucleus (SCN) in the hypothalamus. Multiple types of neurons are involved in the SCN neuronal network (Antle and Silver 2005). In rodents, these mainly include the vasoactive intestinal polypeptide (VIP)-producing and the arginine vasopressin (AVP)-producing neurons (Antle and Silver 2005), and in addition to these, humans also possess neurotensin (NT)-containing neurons (Mai, Kedziora et al. 1991).

Type 2 diabetes mellitus is characterised by hyperglycaemia and insulin resistance. Glucose homeostasis and insulin sensitivity are tightly controlled by the circadian timing system, mainly through balancing sympathetic and parasympathetic outputs from the hypothalamus (Kalsbeek, Yi et al. 2010). Previous studies have shown that impaired insulin secretion in prediabetic animal models results in decreased insulin signalling in the hypothalamus, leading to decreased inhibition of glucose production in the liver and impaired glucose uptake (Schwartz, Woods et al. 2000, Buijs, Guzman-Ruiz et al. 2017). Evidence is accumulating for a link between circadian misalignment, for example, by sleep deprivation, and profound disruptions in blood glucose and insulin levels (Arble, Bass et al. 2015). Thus far, few studies have investigated peripheral clock machinery in individuals with type 2 diabetes (Stenvers, Scheer et al. 2019), and it has never been studied whether the central clock in the SCN itself is affected by type 2 diabetes. The current study aimed to profile and compare SCN neurons, especially the ones producing AVP, VIP and NT, as well as the astroglial cells (using glial fibrillary acidic protein, GFAP, as a marker) and microglia (using ionised calcium-binding adapter molecule 1, IBA1, as a marker) in control and type 2 diabetic individuals.

Methods
Donors Post-mortem hypothalamic tissues from 28 type 2 diabetic and 12 non-diabetic control individuals were obtained from the Netherlands Brain Bank, through autopsy approved by the Medical Ethic Committee of the VU Medical Center, the Netherlands. Individuals with Braak stage V–VI or clinically diagnosed severe dementia were excluded (Braak, Alafuzoff et al. 2006). Sex, age, time/month of death were similar between groups (supplementary material Table 1). Data on the latest post-absorptive blood glucose and HbA1c, although not complete, as indications of glycaemic control are presented in Supplemental Table 1. Other donor details, including post-mortem delay, clinical diagnosis, diagnosed high blood pressure, insulin treatment and cause of death are provided in Supplemental Table 1.
Immunohistochemistry and image analysis

Immunohistochemistry for AVP-ir, VIP-ir, NT-ir, GFAP-ir and IBA1-ir cells in the SCN was performed (see Supplemental Methods). Images were analysed by the Fiji image processing program, an ImageJ distribution (Madison, WI, USA). The soma number and relative intensity of immunoreactivity for AVP-ir, VIP-ir and NT-ir neurons; the number of GFAP-ir astroglial cells and the soma number/soma size for IBA1-ir microglia (per section) were quantified by a blinded investigator (RH) (see Supplemental Methods for further details).

The numbers of AVP-ir and VIP-ir cells at each level of the SCN were plotted along the rostral–caudal axis for all control individuals. To profile the other cells, for each individual we selected consecutive sections next to the one that contained the highest number of AVP-ir cells (Supplemental Fig.1).

Statistics All data are presented as means ± SEM. Comparisons between control and type 2 diabetic individuals were analysed by Student’s t test. A p value of <0.05 was considered to be significant. Daily rhythmicity and monthly variation in the number of AVP-ir, VIP-ir, NT-ir, GFAP-ir and IBA1-ir cells in the SCN was assessed using cosinor analysis SigmaPlot 14.0 software (SPSS, Chicago, IL, USA) (see Supplemental Methods for further details).

Results

AVP-ir neurons were mainly distributed in the dorsal SCN, while VIP-ir neurons were mainly found in the ventral and central SCN (Fig.1, Supplemental Fig.1). NT-ir neurons were visible in the dorsomedial and ventral SCN (Fig.1). When considering all donors (n=40), no correlations were found between the numbers of SCN AVP-ir, VIP-ir and NT-ir neurons and age, post-mortem delay, post-absorptive blood glucose level or HbA1c (Supplemental Fig. 2). Daily rhythmicity and monthly variation in AVP-ir, VIP-ir and NT-ir neurons in the SCN did not reach significance, but acrophase and amplitude in daily rhythmicity were significant in the AVP-ir neurons in non-diabetic control individuals (Supplemental Figs 3, 4). The overall numbers of AVP-ir and VIP, but not NT-ir, neurons, were significantly reduced in the SCN of type 2 diabetic individuals compared with control individuals (Fig. 1). Furthermore, compared with that in the control individuals, the relative intensity of AVP-ir was significantly lower in type 2 diabetic individuals (Fig.1d), indicating a decrease in cellular AVP protein expression in type 2 diabetic individuals. This decrease was not observed for
VIP-ir and NT-ir neurons (Fig. 1 h,l). High blood pressure was diagnosed in a large number of control donors and type 2 diabetic donors, especially those receiving insulin treatment (Supplemental Table 1). Previous studies have found decreased numbers of AVP-ir, VIP-ir and NT-ir neurons in individuals with hypertension (Goncharuk, van Heerikhuize et al. 2001); however, we only found significant reductions in AVP-ir and VIP-ir neurons, but not NT-ir neurons, in individuals (non-diabetic and type 2 diabetic individuals combined) diagnosed with high blood pressure (Supplemental Fig. 5), indicating the reductions in AVP-ir and VIP-ir neurons might be related to insulin resistance rather than high blood pressure. Previous studies also showed a reduction in AVP mRNA expression in the SCN of individuals that had received corticosterone treatment (Liu, Unmehopa et al. 2006). In our study, very few individuals (one non-diabetic donor, four with type 2 diabetes) had received corticosterone treatment. We found no differences in the numbers of AVP-ir, VIP-ir and NT-ir neurons in corticosterone-treated vs non-treated individuals (Supplemental Fig. 6).
Overall, the number of cells showing GFAP immunoreactivity was relatively low (Fig. 2a-d) compared with the number of peptidergic SCN neurons. In some individuals, more commonly, type 2 diabetic individuals, very few GFAP-ir cells were detected in the SCN. GFAP-ir cells were only analysed by cell number. For IBA1-ir microglial cells, the number of soma and the soma size (>20 µm²) were quantified (Fig. 2e-h). When considering all subjects together, no correlation was found between the number of GFAP-ir astroglial cells and IBA1-ir microglial cells in the SCN and age, post-mortem delay, blood glucose level or HbA1c (Supplemental Fig. 2d,e). Daily rhythmicity in the number of GFAP-ir astroglial and IBA1-ir microglial cells did not reach significance (Supplemental Fig. 3). Interestingly, in control individuals, the amplitude of monthly fluctuation of IBA1-ir microglial cell number was more strongly statistically significant than in type 2 diabetic individuals (Supplemental Fig. 4). Similar to the observations for AVP-ir neurons, the number of GFAP-ir cells in the SCN was reduced in type 2 diabetic individuals (Fig. 2d). However, no correlation was found between the number of AVP-ir and GFAP-ir cells (data not shown). The total number of IBA1-ir microglial cells and their average soma size did not differ between control and type 2 diabetic individuals (Fig. 2g,h). Moreover, no difference was found in GFAP-ir astroglial and IBA1-ir microglial cells between individuals with and without high blood pressure (Supplemental Fig. 5). Although both astroglial and microglial cells are involved in neuroinflammation (Streit, Mrak et al. 2004) and therefore could be affected by corticosterone treatment, no difference was found between...
corticosterone-treated and non-treated individuals in terms of numbers of GFAP-ir astroglial and IBA1-ir microglial cells (Supplemental Fig. 6).

**Discussion**

In the current study, we performed an analysis of SCN AVP-ir, VIP-ir and NT-ir neurons and IBA1-ir and GFAP-ir glial cells in post-mortem human brain tissue obtained from non-diabetic and type 2 diabetic individuals. Our analysis revealed that the numbers of AVP-ir neurons, VIP-ir neurons and GFAP-ir astroglial cells is significantly decreased in the SCN of type 2 diabetic individuals.

Some major obstacles currently hamper translational studies on brain dysfunction in type 2 diabetic individuals at the molecular level. First, there is no perfect animal model that fully mimics the pathogenesis of type 2 diabetes in humans. Second, although non-invasive brain imaging techniques have provided data on overall changes in brain metabolism in type 2 diabetes, it is poorly understood what these changes mean for specific brain regions and individual cells. In the current study, the unique collection of the Netherlands Brain Bank, with fully informative medical records, gave us the opportunity to retrogradely analyse the medical characteristics of type 2 diabetic individuals and control subjects, and systemically study differences in their brains at the molecular level.

One of the major targets of the SCN projections is the hypothalamic pre-autonomic neurons (Gomez, Chapleur et al. 1997, Tecleamariam-Mesbah, Kalsbeek et al. 1997). The loss of AVP-ir and VIP-ir SCN neurons, therefore, could result in a disbalanced autonomic hypothalamic output, as often observed in type 2 diabetes (Vinik and Erbas 2013). Intriguingly, the number of GFAP-ir astroglial cells was reduced in the SCN of type 2 diabetic donors, suggesting that astroglial cells play an important role in maintaining SCN function. Indeed, a recent study demonstrated that in the absence of other cellular clocks, the cell-autonomous astroglial intracellular transcription–translation negative feedback loops alone could drive molecular oscillations in the SCN and circadian behaviour in mice (Brancaccio, Edwards et al. 2019).

Previous studies have shown that individuals with type 2 diabetes have a more irregular sleep/wake cycle than the general population (Nakanishi-Minami, Kishida et al. 2012). The ‘cause–effect’ question is whether the reduced number of AVP-ir and VIP-ir neurons and GFAP-ir astroglial cells is responsible for the disturbed sleep/wake rhythms or whether the disturbed sleep/wake rhythms affected the SCN. Observations in elderly people and ageing rats suggest the former, since increasing daytime light exposure not only improved sleep/wake rhythms but also increased AVP-ir in the SCN (Lucassen, Hofman et al. 1995). Nevertheless, whether modifying light exposure can improve the sleep/wake rhythm of individuals with type 2 diabetes and eventually add benefits to their treatment remains to be explored.

In summary, to start to understand the association between circadian clockwork perturbations and the metabolic syndrome in humans, we took advantage of the unique collection of
type 2 diabetes human brain tissue in Netherlands Brain Bank, and systematically analysed SCN cells. Our data indicate that besides regular glucose-lowering medication, normalising circadian rhythms by pharmacological or behavioural approaches might be helpful to treat type 2 diabetes more effectively.

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References


Supplemental material

Methods

Immunohistochemistry

Hypothalamic tissues were emulsion fixed in 10% phosphate-buffered formalin. Fixation time between groups was matched (Ctrl: 50.00±4.2 hours, T2DM: 48.57±2.7 hours, p=0.773). Tissues were paraffin embedded and coronal sectioned to be 6µm. Every 100th section was used for Nissl staining to pre-determine the anatomical orientation of SCN, that was further analysed by the range of AVP-ir and VIP-ir neurons. For immunohistochemistry, two sections proximate to every 100th section of the estimated SCN boundaries were mounted in an aquaest solution on SuperFrost Plus slides and dried at 37 ºC. Sections were first deparaffinised and hydrated, after which they were rinsed in TBS, and were antigen retrieved for 10 min using microwave treatment at 700W in 0.1 M sodium citrate (pH=6.0), sections were then incubated with: 1) rabbit polyclonal anti-AVP or rabbit polyclonal anti-VIP (Netherlands Institute for Brain Research, Amsterdam, the Netherlands) (Van der Woude, Goudsmit et al. 1995), 2) rabbit polyclonal anti-neurotensin (LS-C400910, 1:5000, LifeSpan, LSBio), 3) rabbit polyclonal anti-iba1 (234003, Synaptic Systems, Goettingen, Germany)(Chen, Kelemen et al. 2004), 4) rabbit polyclonal anti-GFAP (N1506, 1:1000, DAKO, USA) (Eggers, Horn et al. 2015), in a TBS solution containing 0.25% gelatin and 0.5% TritonX 100 overnight at 4 ºC; after rinsing in TBS; sections were incubated with biotinylated horse anti-rabbit IgG antibody (1:400, Vector Laboratories) for 60 min, after rinsing, sections were incubated with avidin–biotin complex (1:800, Vectastain Elite ABC kit; Vector Laboratories Inc.) for 60 min; after rinsing again in TBS, sections were incubated in 0.5 mg/ml 3,3’-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) in TBS containing 0.2% ammonium nickel sulfate (DAB/Ni) (BDH; Brunschwig, Amsterdam, The Netherlands) and 0.01%H2O2 (Merck, Darmstadt, Germany) for approximately 15 min. The reaction was stopped in distilled water. The sections were dehydrated in ethanol and delipidized in 100% xylene. Finally, the sections were cover-slipped using Entellan (Merck, Darmstadt, Germany).

Image analysis

Images of all the stained sections were obtained with an EXi Aqua Bio-Imaging Microscopy Camera (EXi Aqua™) (Zeiss Axiovert 200M microscope). Tiled images from the SCN were taken from each subject. These images were analysed in Fiji, an ImageJ distribution. The soma number and the intensity of staining of AVP-ir, VIP-ir and NT-ir neurons and iba1-ir and GFAP-ir glial cells were analysed with their immunoreactivity signals. The DAB/Ni precipitated area was masked and soma size was measured by Fiji. The minimal soma size included in the data analysis was set at 80µm² for neurons (based on the observation that with such a size a nucleus and nucleolus were still visible) and 20µm² for microglia (based on the observation that with such a size a nucleus was visible). The GFAP-ir astroglial cell number
was manually counted due to the dark background of this staining. The soma number and relative intensity of immunoreactivity for AVP-ir, VIP-ir and NT-ir neurons; the number of GFAP-ir astroglial cells and the soma number/soma size for iba1-ir microglia (per section) were quantified by blind analysis.

Statistics

The Time of death and Month of death were matched between groups (Mardia-Watson-Wheeler test). A possible daily rhythmicity (plots with Time of death), and monthly variation (plots with Month of death) for the number of AVP-ir, VIP-ir, NT-ir, GFAP-ir and iba1-ir cells in the SCN was assessed using cosinor analysis with SigmaPlot 14.0 software (SPSS Inc, Chicago, IL, USA). Data were fitted to the following regression: \( y = A + B \cdot \cos(2\pi(x-C)/24) \) for daily rhythmicity, and \( y = A + B \cdot \cos(2\pi(x-C)/12) \) for monthly variation, where \( A \) is the mean level, \( B \) the amplitude and \( C \) the acrophase of the fitted rhythm. An overall p value (main p-value, \( P_m \)) below 0.05 was considered to indicate a significant 24h-rhythmicity or monthly variation (de Goede, Sen et al. 2018).

References


Supplemental figures and figure legends

**Supplemental Figure 1.** The quantification strategy. (a) The distribution of arginine vasopressin immunoreactive (AVP-ir) and vasoactive intestinal polypeptide immunoreactive (VIP-ir) neurons along the rostral to caudal axis of the suprachiasmatic nucleus (SCN) of control subjects. (b) The AVP-ir area in the dorsal part of the SCN is framed by a blue line. (c) The VIP-ir area in the ventral and central parts of the SCN on the consecutive section is framed by a red line. The glial fibrillary acidic protein immunoreactive (GFAP-ir) astroglial cells (d) and the ionized calcium-binding adapter molecule 1 immunoreactive (iba1-ir) microglia (e) are analysed in the merged area framed by the blue and red lines. Data are presented as mean ± SEM. III: third cerebral ventricle. Scale bar: 500μm.
Supplemental Figure 2. Plots of the number of arginine vasopressin immunoreactive (AVP-ir), vasoactive intestinal polypeptide immunoreactive (VIP-ir), neurotensin immunoreactive (NT-ir), glial fibrillary acidic protein immunoreactive (GFAP-ir) and ionized calcium-binding adapter molecule 1 immunoreactive (iba1-ir) cells in the suprachiasmatic nucleus (SCN) according to Age (a1, b1, c1, d1, e1), post-mortem delay (a2, b2, c2, d2, e2), post-absorptive blood glucose (a3, b3, c3, d3, e3), and HbA1c levels (a1, b1, c1, d1, e1). Data for Age and post-mortem delay concern all subjects, i.e., control and T2DM. Data for post-absorptive blood glucose and HbA1c levels only concern some subjects due to limited availability in both control and T2DM.
Supplemental Figure 3. Plots of the number of arginine vasopressin immunoreactive (AVP-ir) (a1-a2), vasoactive intestinal polypeptide immunoreactive (VIP-ir) (b1-b2), neurotensin immunoreactive (NT-ir) (c1-c2), glial fibrillary acidic protein immunoreactive (GFAP-ir) (d1-d2) and ionized calcium-binding adapter molecule 1 immunoreactive (iba1-ir) (e1-e2) cells in the suprachiasmatic nucleus (SCN) according to the time of death of the Ctrl and T2DM subjects (A – C: p values of Mean, Amplitude, and Acrophase of cosinor analysis, respectively, Pm: main P value of rhythmicity).
Supplemental Figure 4. Plots of the number of arginine vasopressin immunoreactive (AVP-ir) (a1-a2), vasoactive intestinal polypeptide immunoreactive (VIP-ir) (b1-b2), neuropeptide Y immunoreactive (NT-ir) (c1-c2), glial fibrillary acidic protein immunoreactive (GFAP-ir) (d1-d2), and ionized calcium-binding adapter molecule 1 immunoreactive (iba1-ir) (e1-e2) cells in the suprachiasmatic nucleus (SCN) according to the month of death of the Ctrl and T2DM subjects (A – C: p values of Mean, Amplitude, and Acrophase of cosinor analysis, respectively, Pm: main P value of rhythmicity).
Supplemental Figure 5. Comparison of arginine vasopressin immunoreactive (AVP-ir) (a), vasoactive intestinal polypeptide immunoreactive (VIP-ir) (b), neurotensin immunoreactive (NT-ir) (c), glial fibrillary acidic protein immunoreactive (GFAP-ir) (d) and ionized calcium-binding adapter molecule 1 immunoreactive (Iba1-ir) (e,f) cells in the suprachiasmatic nucleus (SCN) of the subjects that had high blood pressure (HBP) or had no HBP. Data are presented by mean ± SEM. * P<0.05.
Supplemental Figure 6. Comparison of arginine vasopressin immunoreactive (AVP-ir) (a), vasoactive intestinal polypeptide immunoreactive (VIP-ir) (b), neurotensin immunoreactive (NT-ir) (c), glial fibrillary acidic protein immunoreactive (GFAP-ir) (d) and ionized calcium-binding adapter molecule 1 immunoreactive (iba1-ir) (e,f) cells in the suprachiasmatic nucleus (SCN) of the subjects that received corticosterone (Cort) treatment or did not receive corticosterone treatment. Data are presented by mean ± SEM.
Supplementary Table 1 Clinico-pathological data of patients and matched controls.

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P value 0.008 ns ns 0.490 0.727 0.063 0.154

PMD: post-mortem delay between time of death and time of autopsy (hours), TOD: time of death, MOD: Month of death, HBP: high blood pressure, CVA: cerebrovascular accident. TIA: transient ischemic attack, ns: not significant (by Mardia-Watson-Wheeler test). P value was obtained by Tukey's multiple comparison test following one-way ANOVA analysis.
Chapter 4

The association between type 2 diabetes and cholinergic neurons in the human nucleus basalis of Meynert

In preparation

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4. Department of Neuroscience, Faculty of Health, Medicine and Life Sciences, Maastricht University, Maastricht, The Netherlands.
5. Department of Translational Neuroscience, University Medical Center Utrecht Brain Center, Utrecht University, Utrecht, the Netherlands.
Abstract
Type 2 diabetes (T2DM) increases the risk of Alzheimer’s disease (AD), and proper anti-diabetic treatment attenuates AD development. An important hallmark of AD is the formation of neurofibrillary tangles (NFT). One of the first areas where these NFTs form is the nucleus basalis of Meynert (NBM), resulting in a disturbed function of the residing cholinergic neurons. This disturbance in the functioning of the cholinergic neurons results in cognitive decline. Microglia might play an important role in the mechanism underlying the development of neuronal dysfunction in AD. Interestingly, microglia dysfunction has also been implicated to underlie T2DM development, which might be an important link between these two diseases. So far, it is completely unclear whether any changes in NBM cholinergic neurons and microglia are detectable in the T2DM brain before the onset of cognitive decline. Furthermore, how anti-diabetic treatments affect the cholinergic neurons and microglia in the NBM is not known. In the current study we investigated the NBM of control and T2DM subjects at different stages of cognitive decline. We performed an immunohistochemical analysis of acetylcholine transferase (ChAT, a marker for cholinergic neurons), ionized calcium-binding adapter molecule 1 (Iba1, a marker for microglia) and the tau protein using three different antibodies recognizing early, mid and late stage of tau pathology. With the progression of cognitive decline the amount of ChAT-ir decreased and pathological changes of the tau protein increased. Remarkably, T2DM subjects without cognitive decline and treated with insulin showed significantly higher ChAT-ir and less pathological tau changes as compared to T2DM subjects without insulin treatment. Cognitive decline did not associate with any changes in microglia, but T2DM decreased the number of microglia in the NBM of subjects without cognitive decline. Our results hint towards a protective effect of insulin treatment on the cholinergic neurons of T2DM subjects without cognitive decline.
Introduction

Type 2 diabetes (T2DM) is associated with the development of Alzheimer’s disease (AD). Poor glycemic control seems to accelerate cognitive decline in AD patients, whereas controlling glycemic levels with diabetes medication slows down the progression of dementia (Yaffe, Falvey et al. 2012). Moreover, while T2DM patients have an increased risk to develop AD, the cognitive decline in AD progresses slower in individuals with T2DM compared to those without T2DM (Biessels, Staekenborg et al. 2006, Cheng, Huang et al. 2012, Davis, Zilkens et al. 2017). Diabetes treatment seems to play a role here. Patients treated with a combination of insulin and other diabetes medication showed markedly less tau pathology (Beeri et al. 2008), one of the hallmarks of AD. In another study, insulin-treated T2DM patients with AD showed slower clinical decline than those treated with oral diabetes medication instead (Plastino, Fava et al. 2010). Together indicating that insulin may slow down the progression of cognitive decline.

During AD development, the cholinergic neurons in the nucleus basalis of Meynert (NBM) are one of the first to be affected. These neurons provide the cortex with cholinergic innervation and a disturbance in this innervation results in cognitive dysfunction. With the progression of AD, the number of cholinergic neurons in the NBM drastically decreases (Zhu, Unmehopa et al. 2016). This neuronal loss is highly correlated with increased tau pathology, indicating tau pathology is closely associated with NBM dysfunction (Zhu, Unmehopa et al. 2016).

Even though it is well known that T2DM increases the risk of AD, little is known about the effect of T2DM on NBM function. Whether changes in cholinergic neurons and tau pathology are detectable in the T2DM brain, maybe even before the onset of cognitive decline, is completely unknown. Furthermore, whether the beneficial effects of anti-diabetic treatment on cognitive decline are reflected in the preservation of NBM function is also unclear. The detection of changes in the pre-demented brain of T2DM subjects could be of great value, as they might shed some light on the T2DM-induced pathological changes that lead to AD. Ultimately, such observations might reveal therapeutic targets for the prevention of cognitive dysfunction, particularly in those with metabolic disorders. Therefore, we set out to investigate, in post-mortem human brain tissue, the changes of cholinergic neuron and tau pathology in the NBM of T2DM patients, with or without mild and severe dementia.

Material & Methods

Subject information

All brain material was obtained from the Netherlands Brain Bank. The donor or their next of kin gave informed consent for a brain autopsy and for the use of the brain material and medical records for research purposes. In total, 63 post-mortem human NBM samples were studied. A control group of 19 subjects without T2DM and no cognitive decline (ncd-CTRL), 22 subjects diagnosed with T2DM but no cognitive decline (ncd-T2DM), 14 subjects diagnosed
with T2DM combined with moderate cognitive decline (mcd-T2DM), and 7 subjects with T2DM and severe cognitive decline (scd-T2DM) (Supplementary table 1). Among the ncd-T2DM subjects, 10 subjects received insulin treatment and the other 12 subjects did not (Supplementary table 1). The groups were matched in order to prevent any effect of possible confounding factors. The ncd-CTRL and ncd-T2DM subjects matched for gender, age, BMI, post absorptive glucose, HbA1c, brain weight, fixation time and Braak stage (Table 1). In the T2DM subjects, the three different stages of cognitive decline matched for almost all factors, except for brain weight and Braak stage (Table 1), which could be subscribed to (the known effect of) severe cognitive decline. The ncd-T2DM subjects treated with insulin matched with the ncd-T2DM subjects without insulin treatment for all confounding factors (Table 1). Data on the latest post-absorptive blood glucose and HbA1c, a measure of 3-month average glucose levels, although incomplete, are also presented in Table 1. An overview of more details of each individual subject, medication use, clinical diagnosis and cause of death is provided in Supplementary Table 1.

**Histology**

After autopsy, the isolated hypothalamic tissues were immediately immersed in formalin and fixed at room temperature for one to two months (Supplementary Table 1). Tissues were then ethanol-dehydrated, toluene-cleared and paraffin-embedded. Six μm sequential hypothalamic sections were sectioned along the rostro-caudal axis from the lamina terminalis to the mammillary bodies. The anatomical orientation and rostro-caudal range of the NBM was determined by thionin staining. Sections were mounted on glass slides (superfrost+) and dried on a 37°C heating plate. After 48 hours, sections were deparaffinized in 100% xylene, rehydrated in grading ethanol (100% - 50%) and rinsed in distilled water. Next, the sections were submerged in a 0.5% thionin solution for 5 minutes and rinsed in water. After dehydration in graded ethanol (50% - 100%) and xylene, sections were coverslipped using Entellan® (Sigma-Aldrich, 107960), dried by air and ready for analysis.

**Morphometry of the Nucleus Basalis of Meynert**

Because the NBM consists of a very large cell population, we performed our measurements in a standardized part of the NBM which was described in previous studies (Mesulam, Mufson et al. 1983, Dubelaar, Verwer et al. 2004, Zhu, Unmehopa et al. 2016), i.e. the medial (Ch4-am) and lateral subdivisions (Ch4-lm) of the anterior NBM at the level of the fornix, and/or the anterior commissure. After this standardized part of the NBM (Figure 1) was located in the thionin stained section, 12 consecutive sections were mounted for each subject to perform immunohistochemistry on.
Table 1. Subject group characteristics.

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BMI: body mass index, HbA1c: glycated hemoglobin. Data was analyzed using a Mann-Whitney test for two groups and a Kruskal-Wallis test for 3 groups. Gender differences were calculated using Chi-square test. Data on BMI, post absorptive glucose and HbA1c were incomplete preventing reliable statistical testing in some cases.
**Immunohistochemistry**

The sections used for immunohistochemistry were acquired in an identical way as described in the histology section above, but instead of submerging them in a thionin solution we continued with the following procedures. For the acetylcholine transferase (ChAT) and ionized calcium binding adaptor molecule 1 (Iba1) antibodies we performed heat-induced epitope retrieval by submerging the sections in citrate buffer (pH 6.0) and heated them for 10 minutes by microwave treatment (700 W), and let the sections cool down for 30 minutes. The AT8 antibody did not need antigen retrieval, but for the CP13 and PHF1 antibody we performed room temperature induced epitope retrieval by submerging the sections in formic acid (pH 2.0) for 10 minutes. After these procedures, sections were washed in TBS, and incubated with the primary antibody overnight at 4°C. The AT8 antibody was diluted in SUMI-milk (5% nonfat milk, 0.25% gelatin, 0.5% Triton X-100 in TBS), the rest of the antibodies were diluted in normal SUMI (no milk).

The next day, sections were washed in TBS, incubated for 60 minutes with biotinylated horse anti-mouse IgG antibody (1:400, Vector Laboratories), washed in TBS, incubated for 60 minutes with avidin–biotin complex (1:800, Vectastain Elite ABC kit; Vector Laboratories Inc.) and washed in TBS. Finally, sections were incubated in 0.5 mg/ml 3,3'-Diaminobenzidine (Sigma Chemical Co., St. Louis, MO) in TBS (DAB), for ChAT 0.2% ammonium nickel sulfate was added (DAB/Ni) (BDH; Brunschwig, Amsterdam, The Netherlands) and 0.01% H2O2 (Merck, Darmstadt, Germany) for 20 min. The reaction was stopped in distilled water. Afterwards, sections were ethanol dehydrated, xylene cleared and Entelan cover slipped. Primary antibodies used were: ChAT (Sigma Aldrich, USA, AB144P), AT8 (ThermoFisher, USA, MN1020) and Iba-1 (Synaptic Systems, USA, 234 003). The antibodies against CP13 and PHF-1 were a kind gift from Professor F. van Leeuwen (Petry et al. 2014).

**Quantitative analysis**

An investigator blind to the subject information carried out the quantitative image analyses. In the thionin-stained sections, neuron density (the nucleolus-containing neurons/mm$^3$) in the NBM was calculated with Image Pro version 6.3 (Media Cybernetics). This procedure was similar to what has been described before by Dubelaar et al. (Dubelaar, Verwer et al. 2004) with minor adjustments. In short, using the 5-x objective, an overview image of the section was made and the NBM was manually outlined. The image analysis software covered this outline with a grid dividing it in several fields of view using the 40-x objective. We counted the nucleolus containing cells in at least 40 fields of view; subsequently the software calculated the neuron density based on neuron count, area of the outline and the section thickness. The number of nucleolus containing ChAT-ir cells was counted using the same method. Quantification of the integrated optical density (IOD) of immunocytochemistry signals was also described in a previous study (Zhu et al. 2016). An outline of the NBM was drawn manually at 10x magnification and the threshold for a positive signal was set at three times the optical density.
(OD) of the background. The software then calculated the average OD of the positive signal and the relative surface area of the outline covered by positive signal (area mask). By multiplying the average OD with the area-masked we calculated the integrated optical density (IOD). For the quantification of the number and size of iba1-ir microglial soma we performed the analysis reported in a previous study (Kalsbeek et al. 2020).

**Statistics**

We performed Kruskal-Wallis tests to identify any group differences. Post-hoc testing was performed to identify any differences between any two separate groups. We controlled for multiple testing by correcting for false discovery rate using the Benjamini-Krieger and Yeketiel criterion (Benjamini, Krieger et al. 2006), resulting in an adjusted p-value (q-value). The insulin treatment associated effect was investigated using Mann-Whitney testing. Correlations were measured using linear regression. A p-value or q-value ≤ 0.05 was considered statistically significant. All statistical tests were performed using GraphPad Prism 8.12.

**Results**

*Neuron density in the NBM of T2DM subjects is stable during cognitive decline*

As cognitive decline is associated with neuronal atrophy of the NBM (Zhu, Unmehopa et al. 2016), we investigated the neuron density in T2DM subjects at different stages of cognitive decline. We counted the number of nucleolus-containing neurons per delineated NBM area (neuron density) in the thionin stained section (Figure 2 A-H). These stainings were suitable for localization of the standardized NBM area and for making the distinction between nucleolus containing neurons and glia cells. First, we compared ncd-T2DM subjects with matched ncd-CTRL subjects and found no differences in neuron density of the NBM (Figure 2I). The T2DM subjects at three different stages of cognitive decline also did not show any differences in neuron density (Figure 2J). We also investigated a possible insulin-associated effect on
Figure 2. Neuron density in the NBM of T2DM subjects is stable during cognitive decline. (A - H) Thionin positive neurons in the nucleus basalis of Meynert (NBM) of a ncd-CTRL subject (A), a ncd-T2DM subject (B), mcd-T2DM subject (C) and scd-T2DM subject (D) (higher magnification of the areas framed in A - D are shown in E - H respectively). Comparisons of the thionin positive neuron density between ncd-CTRL and ncd-T2DM subjects (I), between the three different stages of cognitive decline in T2DM subjects (J) and between insulin treatment-associated effect in ncd-T2DM subjects (K). ON: optic nerve and arrow heads indicate a nucleolus. Scale bars are 1 mm in D, and 100 µm in H. Data is represented as mean ± SEM. Significance calculated using a Mann-Whitney test in I and K, and a Kruskall-Wallis test in J. The p-values from post-hoc analysis was corrected for multiple comparisons using the Benjmini-Krieger and Yekutieli criterion.

neuron density within the ncd-T2DM group, but found no differences between subjects with or without insulin treatment (Figure 2K). Together this shows that in T2DM subjects, contrary to previous research in non-T2DM subjects (Zhu, Unmehopa et al. 2016), the total number of neurons remains stable during cognitive decline, indicating a protective effect of T2DM.

Insulin treatment is associated with increased metabolic activity of cholinergic neurons in the NBM of T2DM subjects

For the visualization of the cholinergic neurons in the NBM, we used an antibody against acetylcholine transferase (ChAT). Most of the neurons in the NBM showed ChAT expression in both their cell body and fibers (Figure 3 A-H). The number of ChAT-ir neurons relative to the number of thionin-stained neurons in the NBM showed a positive correlation with brain weight in ncd-CTRL and mcd-T2DM subjects (Supplementary figure 1). However, because these groups matched for brain weight (Table 1), this correlation did not influence our results. Because the total number of neurons was similar between groups, we neither expected any
Figure 3. Insulin treatment is associated with an increased amount of ChAT-ir in the NBM of ncd-T2DM subjects. (A - H) Acetyl choline transferase immunoreactive (ChAT-ir) neurons in the nucleus basalis of Meynert (NBM) of a ncd-CTRL subject (A), a ncd-T2DM subject (B), mcd-T2DM subject (C) and an scd-T2DM subject (D) (higher magnification of the areas framed in A - D are shown in E - H respectively). Comparisons of the relative number of ChAT-ir neurons between: ncd-CTRL and ncd-T2DM subjects (I), three different stages of cognitive decline in T2DM subjects (J) and between ncd-T2DM subjects with or without insulin treatment (K). Comparisons of ChAT-ir integrated optical density between: ncd-CTRL and ncd-T2DM subjects (L), three different stages of cognitive decline in T2DM subjects (M) and between ncd-T2DM subjects with or without insulin treatment (N). ON: optic nerve and arrow heads indicate a nucleolus. Scale bars are 1 mm in D, and 100 µm in H. Data is represented as mean ± SEM. Significance calculated using a Mann-Whitney test in I, K, L and N and a Kruskall-Wallis test in J and M. The p-values from post-hoc analysis was corrected for multiple comparisons using the Benjamini-Krieger and Yekutieli criterion. * P < 0.05 and ** P < 0.01.

differences in the percentage of ChAT-ir neurons. Indeed, we did not find any differences between ncd-CTRL and ncd-T2DM (Figure 3I), nor between cognitive decline groups (Figure 3J) and we also did not observe an insulin-associated effect (Figure 3K). We next used the integrated optical density (IOD) of the ChAT-ir signal to investigate the metabolic activity of the ChAT-ir neurons as done previously (Dubelaar, Mufson et al. 2006). None of the confounding factors showed a correlation with the IOD of ChAT-ir (Supplementary figure 2). In the ncd subjects,
the IOD of ChAT-ir was not different between CTRL and T2DM subjects (Figure 3L). In the T2DM subjects, the three cognitive decline groups showed a significant difference (Figure 3M, Kruskall-Wallis, p = 0.010). Post hoc analysis revealed that ChAT-ir was dramatically lower in both mcd-T2DM and scd-T2DM compared to ncd-T2DM (q = 0.016 and q = 0.008, respectively). Interestingly, within the ncd-T2DM subjects, we found that insulin treatment associated with an increase of ChAT-ir (Figure 3N, Mann-Whitney, p = 0.048). Together these results indicate that insulin treatment enhances the metabolic activity of ChAT-ir neurons in the NBM of T2DM subjects.

**Insulin treatment protects against early changes in tau pathology.**

To investigate any pathological changes in the tau protein, we first used the antibody CP13, commonly used to detect a relatively early stage (pre-tangle) of tau pathology (Petry, Pelletier et al. 2014). The expression of CP13 was present within the cell bodies and also in the fibers (Figure 4 A-H). The expression of CP13 showed a significant positive association to age, only in ncd-CTRL and ncd-T2DM subjects (Supplementary figure 2). Because these two groups were matched for age (Table 1), these correlations did not affect our results. It does, however, show that pathological changes in tau develop with age, even in subjects without cognitive decline. Within the ncd subjects there was no difference between CTRL and T2DM subjects (Figure 4I). As expected, there was a clear difference in CP13-ir among the cognitive decline subjects (Figure 4J, Kruskal-Wallis, p = 0.0002). The amount of CP13-ir gradually increased with the severity of cognitive decline and post hoc analysis showed that CP13-ir was significantly increased in both mcd-TDM and scd-T2DM subjects compared to ncd-T2DM subjects (Figure 4J, q = 0.005 and q = 0.0001, respectively). Additionally, CP13-ir in the scd-T2DM subjects was significantly higher compared to the mcd-T2DM subjects (Figure 4J, q = 0.034). Intriguingly, within the ncd-T2DM subjects we found an insulin treatment associated effect on CP13-ir; the subjects who received insulin treatment had significantly less CP13-ir compared to those which did not receive insulin treatment (Figure 4J, Mann-Whitney, p = 0.011). Together this indicates that insulin treatment in T2DM subjects inhibits the early pathological changes in tau phosphorylation as identified by the CP13-ir.

Next, we checked PHF-1-ir, which recognizes tau changes that occur in a later stage of cognitive decline than those recognized by CP13-ir (Petry, Pelletier et al. 2014). The pattern of expression was similar to- but more extensive than- that of CP13 (Figure 5 A-H). Again, we found a significant positive correlation with age, but this time only in ncd-T2DM subjects (Supplementary Figure 4). Both ncd-CTRL and ncd-T2DM subjects showed very little PHF-1-ir and were not significantly different (Figure 5I). Similar to CP13, we found that PHF-1-ir gradually increased as cognitive decline progressed in T2DM subjects (Figure 5J, Kruskal-Wallis, p < 0.0001). Post hoc analysis showed that the scd-T2DM subjects had significantly higher PHF-1-ir compared to ncd-T2DM and mcd-T2DM and that mcd-T2DM subjects had
Figure 4. Insulin treatment is associated with decreased CP13-ir in ncd-T2DM subjects. (A - H) CP13 immunoreactivity (CP13-ir) in the nucleus basalis of Meynert (NBM) of a ncd-CTRL subject (A), a ncd-T2DM subject (B), mcd-T2DM subject (C) or scd-T2DM subject (D) (higher magnification of the areas framed in A - D are shown in E - H respectively). Comparisons of the integrated optical density of CP13-ir between: ncd-CTRL and ncd-T2DM subjects (I), three different stages of cognitive decline in T2DM subjects (J) and between ncd-T2DM subjects with or without insulin treatment (K). ON: optic nerve. Scale bars are 1 mm in D, and 100 µm in H. Data is represented as mean ± SEM. Significance calculated using a Mann-Whitney test in I and K and a Kruskall-Wallis test in J. The p-values from post-hoc analysis was corrected for multiple comparisons using the Benjamini-Krieger and Yekutieli criterion. * P < 0.05, ** P < 0.01 and *** P < 0.001.

significantly higher PHF-1-ir compared to ncd-T2DM (q < 0.0001, q = 0.026 and q = 0.009, respectively). For PHF-1-ir we did not find an insulin treatment associated effect within the ncd-T2DM subjects (Figure 5K).

Finally we used the classical tau-marker AT8, which recognizes pathological changes in tau protein occurring in a relatively late stage of cognitive decline (Petry, Pelletier et al. 2014). AT8 expression was mostly present in the cell bodies, but also some fibers were positive (Figure 6 A-H). Like the previous two tau-markers, AT8-ir showed a significant positive correlation with age, only in ncd-CTRL (Supplementary figure 4). And again, we found no differences between the ncd-CTRL and the ncd-T2DM subjects (Figure 6I). Additionally, we found AT8-ir to gradually increase with cognitive decline (Figure 6J, Kruskal-Wallis, P < 0.0001). Post hoc analysis showed that AT8-ir was highest in the scd-T2DM group, significantly higher compared to ncd-T2DM and mcd-T2DM groups (q < 0.0010 and q = 0.040, respectively). Furthermore, AT8-ir in the mcd-T2DM group was significantly higher compared to the ncd-T2DM group as well (q = 0.001). Within the ncd-T2DM subjects insulin treatment was associated with decreased
Figure 5. PHF1-ir is increased in T2DM subjects with moderate and severe cognitive decline. (A - H) PHF1 immunoreactivity (PHF1-ir) in the nucleus basalis of Meynert (NBM) of a ncd-CTRL subject (A), a ncd-T2DM subject (B), mcd-T2DM subject (C) or scd-T2DM subject (D) (higher magnification of the areas framed in A - D are shown in E - H respectively). Comparisons of the integrated optical density of PHF1-ir between: ncd-CTRL and ncd-T2DM subjects (I), three different stages of cognitive decline in T2DM subjects (J) and between ncd-T2DM subjects with or without insulin treatment (K). ON: optic nerve. Scale bars are 1 mm in D, and 100 µm in H. Data is represented as mean ± SEM. Significance calculated using a Mann-Whitney test in I and K and a Kruskall-Wallis test in J. The p-values from post-hoc analysis was corrected for multiple comparisons using the Benjamini-Krieger and Yekutieli criterion. * P < 0.05, ** P < 0.01 and *** P < 0.001.

AT8-ir, although this effect just missed significance (Figure 6K, Mann-Whitney, p = 0.057). All together this shows that the pathological changes in tau protein already increase with age before the onset of cognitive decline. Insulin treatment seems to decelerate this process, thereby possibly also slowing down cognitive decline.

The number of microglia in the NBM is downregulated in ncd-T2DM subjects, without further changes with cognitive decline

As microglia have been proposed to play an active role in the obesity-associated cognitive decline found in mice (Cope et al. 2018), we investigated the iba1-ir microglia in our human samples. Interestingly, we found a strong positive correlation in ncd-T2DM subjects between BMI and the number of iba1-ir microglia (Supplementary figure 4). Because we matched for BMI (Table 1), this correlation did not affect our results. The number and size of iba1-ir soma did not show a correlation with any other confounding factors in any other group (Supplementary figure 4).
Figure 6. AT8-ir gradually increases with cognitive decline in T2DM subjects. (A - H) AT8 immunoreactivity (AT8-ir) in the nucleus basalis of Meynert (NBM) of a ncd-CTRL subject (A), a ncd-T2DM subject (B), mcd-T2DM subject (C) or scd-T2DM subject (D) (higher magnification of the areas framed in A - D are shown in E - H respectively). Comparisons of the integrated optical density of PHF-1-ir between: ncd-CTRL and ncd-T2DM subjects (I), three different stages of cognitive decline in T2DM subjects (J) and between ncd-T2DM subjects with or without insulin treatment (K). ON: optic nerve. Scale bars are 1 mm in D, and 100 µm in H. Data is represented as mean ± SEM. Significance calculated using a Mann-Whitney test in I and K and a Kruskall-Wallis test in J. The p-values from post-hoc analysis was corrected for multiple comparisons using the Benjamini-Krieger and Yekutieli criterion. * P < 0.05, ** P < 0.01 and *** P < 0.001.

We found a clear difference in the number of iba1-ir microglia in the subjects with no cognitive decline; ncd-T2DM subjects had significantly less iba1-ir microglia compared to ncd-CTRL subjects (Figure 7I, Mann-Whitney, p = 0.034). Surprisingly, this decrease in microglia number did not continue with cognitive decline, as we found no differences among the T2DM subjects with different stages of cognitive decline (Figure 7J).

Insulin-treatment did not associate to the number of iba1-ir microglia neither (Figure 7K).

Contrary to iba1-ir microglial cell number, we did not observe any difference in the size of the iba-ir microglial somata; not between ncd-CTRL and ncd-T2DM (Figure 7L), not in the different cognitive decline subjects (Figure 7M) and not between insulin treated and non-treated ncd-T2DM subjects (Figure 7N).

Together these data show that, in humans, cognitive decline is not associated with increased microglial activity. It’s rather BMI in a T2DM setting that affects microglia in the NBM, even before the onset of cognitive decline.
Figure 7. Iba1-ir microglia number is decreased in the NBM of ncd-T2DM compared to ncd-CTRL subjects. (A - H) Ionized calcium-binding adapter molecule 1 immunoreactivity (iba1-ir) in the nucleus basalis of Meynert (NBM) of a ncd-CTRL subject (A), a ncd-T2DM subject (B), mcd-T2DM subject (C) or scd-T2DM subject (D) (higher magnification of the areas framed in A - D are shown in E - H respectively). Comparisons of the number of iba1-ir microglial soma between: ncd-CTRL and ncd-T2DM subjects (I), three different stages of cognitive decline in T2DM subjects (J) and between ncd-T2DM subjects with or without insulin treatment (K). Comparisons of the average size of iba1-ir microglial soma between: ncd-CTRL and ncd-T2DM subjects (L), three different stages of cognitive decline in T2DM subjects (M) and between ncd-T2DM subjects with or without insulin treatment (N). ON: optic nerve. Scale bars are 1 mm in D, and 100 µm in H. Data is represented as mean ± SEM Significance calculated using a Mann-Whitney test in I and K and a Kruskall-Wallis test in J. The p-values from post-hoc analysis were corrected for multiple comparisons using the Benjamini-Krieger and Yekutieli criterion. * P < 0.05.

Discussion
To understand the mechanistic link between T2DM and cognitive decline, we studied neurons and microglia in the NBM of control subjects and T2DM subjects with or without clinically or pathologically diagnosed dementia. We found no difference in ChAT-ir, CP13-ir, PHF1-ir and AT8-ir between CTRL subjects and all T2DM subjects. However, when sub-dividing the T2DM subjects, we found that those treated with insulin had significantly more ChAT-ir and less CP13-ir as compared to the T2DM subjects without insulin treatment, suggesting a neuroprotective effect of insulin treatment in T2DM. Interestingly, we also observed a
significant reduction in the amount of iba1-ir microglia in the T2DM subjects without cognitive decline (ncd-T2DM) as compared to CTRL subjects without cognitive decline (ncd-CTRL) (Fig.7I). However, this decrease of microglia cell number did not continue with a more severe cognitive decline. These data suggest that in the NBM, microglial changes take place before the neuronal changes do.

Neuronal loss in the NBM is a well-established characteristic of AD (Arendt, Bigl et al. 1985, Cullen, Halliday et al. 1997). Our results show that the total number of neurons and the number ChAT-ir neurons in the NBM are relatively stable in T2DM, a significant decrease of ChAT IOD is only found at the later severe stage of AD, together with the increased tau pathology. Therefore, our findings are in alignment with previous research, and the question whether NBM neurons are more affected in T2DM due to the diabetic pathogenesis has to be refuted by the current data. However, to our surprise, we did observe a clear loss of iba1-ir microglial cells in the NBM. This raises the very interesting question on how microglial activity can be affected heterogeneously in different brain regions. In our recent studies on neurons and glial cells in the infundibular (IFN) and suprachiasmatic nuclei (SCN) in T2DM subjects, we could not detect any significant differences in iba1-ir and/or TMEM119-ir microglia between the non-diabetic control subjects and the T2DM patients (Hogenboom, Kalsbeek et al. 2019, Kalsbeek et al. 2020). IFN and SCN are important brain regions in the hypothalamic control of food intake, body weight and biological rhythms, whereas the extra-hypothalamic NBM is important in the control of learning and memory. These data thus suggest that microglial activity can be significantly affected outside of the hypothalamus while well-maintained inside the hypothalamus. Indeed, via a sophisticated genetic approach, the Prinz lab showed that under different physiological and pathological circumstances microglia may respond differently depending on the brain region involved (Tay, Mai et al. 2017, Masuda, Sankowski et al. 2019). Our next goal is to further dissect the intracellular mechanism underlying these intra- and extra-hypothalamic differences in microglia.

In the healthy CNS environment, microglia make up ~10% of the cells in the brain and are engaged in surveillance and homeostatic regulation and remodelling of neuronal activity (Li, Du et al. 2012). Microglia are considered to be the primary sensors of pathological changes in the CNS (Prinz and Priller 2014). Furthermore, microglia are the first cell type to respond to injury by transforming from their surveillance state into an activated form, which enables them to migrate, proliferate and actively communicate with neighbouring neurons and non-neuronal cells (Kettenmann, Hanisch et al. 2011, Kettenmann, Kirchhoff et al. 2013). The latter is achieved by the release of various cytokines and chemokines. Due to the technical limitations of our study-design using post-mortem human brain tissue, we could not detect changes in cytokine or chemokine production in the microglia. Nevertheless, we expect that cytokine and chemokine production, as well as microglial immunometabolism are also affected in these microglia. One possible consequence of declined microglial activity is insufficient
immune surveillance and phagocytosis, which can be a causal factor eventually leading to neurodegeneration in the T2DM NBM.

In our study, we also observed a higher ChAT-ir IOD in insulin-treated T2DM subjects. It is well known that insulin not only regulates blood glucose levels, but is also one of the major circulating hormones that provides metabolic feedback to the brain. The bulk of evidence suggests that insulin-signalling pathways play an important role in neural growth and protection (Recio-Pinto, Lang et al. 1984, Holscher 2014, Aghanoori, Smith et al. 2017). However, it is still unclear how insulin exactly enters the brain, which brain cells are the acting targets of insulin, and which multi-faceted roles insulin plays in the different brain cells. Our study for the first time investigated the involvement of impaired insulin signaling in the crosslink between T2DM and AD, by looking at neurons and glial cells in the NBM.

The preservation of ChAT-ir neurons in insulin-treated subjects could be caused by a direct insulin action on ChAT neurons. It has been reported that insulin stimulates Fos expression in ChAT-expressing neurons in the brainstem (Senthilkumaran, Bobrovskaya et al. 2018), and lack of insulin in the developing brain has been associated with impairment of ChAT gene expression (Abbasi, Behnam-Rassouli et al. 2018). However, we cannot exclude an indirect peripheral effect of insulin through the lowering of blood glucose, stabilizing blood glucose fluctuation or changing other circulating metabolic hormones and metabolites. Additionally, insulin could also indirectly affect ChAT neurons by acting on neighboring non-neuronal cells such as microglia, astrocytes and endothelial cells (Zhang, Chen et al. 2014, Zhang, Sloan et al. 2016), which also express insulin receptors. Consequently, non-neuronal glial cells could also exert a beneficial role in maintaining ChAT neuronal function. In vivo or in vitro models employing brain regional and cell specific tools to mechanistically study insulin action in ChAT cells will be necessary to answer these questions.

Different brain cells have different intracellular insulin signaling pathways. One major remaining question in T2DM is whether brain insulin resistance is due to similar cellular mechanism as peripheral insulin resistance. A further comprehensive mapping of brain insulin action and insulin resistance will be necessary to determine whether targeting the insulin signaling pathway can also be used to develop pharmacotherapies for the treatment of AD.

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References


Kalsbeek et al. (2020). “Insulin treatment protects against pathological changes associated with cognitive decline in the brain of type 2 diabetics individuals.” JCI Insight In press.


Supplementary Figures

Supplementary Figure 1. Plots of thionin-positive neuron density according to body mass index (BMI, kg/m²), age and brain weight in ncd-CTRL, ncd-T2DM, mcd-T2DM and scd-T2DM subjects (A-C, D-F, G and H, and I and J, respectively). Plots of acetylcholine transferase immunoreactive (ChAT-ir) neuron density according to BMI, age and brain weight in ncd-CTRL, ncd-T2DM, mcd-T2DM and scd-T2DM subjects (K-M, N-P, Q and R, and S and T, respectively).
Supplementary Figure 2. Plots of acetylcholine transferase immunoreactive (ChAT-ir) neuron integrated optical density (IOD) according to body mass index (BMI, kg/m²), age and brain weight in ncd-CTRL, ncd-T2DM, mcd-T2DM and scd-T2DM subjects (A-C, D-F, G and H, and I and J, respectively). Plots of CP13-ir neuron IOD according to BMI, age and brain weight in ncd-CTRL, ncd-T2DM, mcd-T2DM and scd-T2DM subjects (K-M, N-P, Q and R, and S and T, respectively).
Supplementary Figure 3. Plots PHF-1 immunoreactive (PHF-1-ir) neuron integrated optical density (IOD) according to body mass index (BMI, kg/m²), age and brain weight in ncd-CTRL, ncd-T2DM, mcd-T2DM and scd-T2DM subjects (A-C, D-F, G and H, and I and J, respectively). Plots of AT8-ir neuron IOD according to BMI, age and brain weight in ncd-CTRL, ncd-T2DM, mcd-T2DM and scd-T2DM subjects (K-M, N-P, Q and R, and S and T, respectively).
Supplementary Figure 4. Plots of ionized calcium-binding adapter molecule 1 immunoreactive (iba1-ir) microglial number according to body mass index (BMI, kg/m²), age and brain weight in ncd-CTRL, ncd-T2DM, mcd-T2DM and scd-T2DM subjects (A–C, D–F, G and H, and I and J, respectively). Plots of the average iba1-ir microglial soma size according to BMI, age and brain weight in ncd-CTRL, ncd-T2DM, mcd-T2DM and scd-T2DM subjects (K–M, N–P, Q and R, and S and T, respectively).
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- Septic syndrome after complicated aorta aneurysm
- Respiratory insufficiency, metastasised cervix carcinoma
- Pneumonia, myocard infarct, dyspnoe
- Mesenterial ischemia complications, dyspnea, atrial fibrillation
- Respiratory insufficiency, cardiac shock, cholecystolithiasis, hypothyroidism
- Cardiac arrest, cardiac diseases
- Kidney failure, dehydration, Heart failure, renal insufficiency.
- Heart attack, Ischemic heart disease, kyphosis of backbone.
- Cachexia, cardiac failure, Encephalopathy, mitral valve insufficiency.
- Pulmonary metastasis of vulva carcinoma.
- Heart failure, prostate carcinoma
- Acute myocardial infarction, prostate carcinoma.
- Bronchopneumonia, cystitis, pyelitis, CVA, myocardial infarction
- Cardiac decompensation, refluxesophagitis
- Dehydration, angina pectoris
- Respiratory failure, angina pectoris, mitralis valve insufficiency
- Heart failure, metastatic breast cancer, scoliosis.
- Heart failure, cachexia and dehydration, pulmonary disease
- Legal euthanasia, Ischemic colitis, heart failure with dyspnoea.
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PMD: post-mortem delay between time of death and time of autopsy (hours), BMI: body mass index, CVA: cerebrovascular incident
Chapter 5

Microglia energy metabolism in metabolic disorder


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Abstract

Microglia are the resident macrophages of the CNS, and are in charge of maintaining a healthy microenvironment to ensure neuronal survival. Microglia carry out a non-stop patrol of the CNS, make contact with neurons and look for abnormalities, all of which requires a vast amount of energy. This non-signaling energy demand increases after activation by pathogens, neuronal damage or other kinds of stimulation. Of the three major energy substrates – glucose, fatty acids and glutamine – glucose is crucial for microglia survival and several glucose transporters are expressed to supply sufficient glucose influx. Fatty acids are another source of energy for microglia and have also been shown to strongly influence microglial immune activity. Glutamine, although possibly suitable for use as an energy substrate by microglia, has been shown to have neurotoxic effects when overloaded. Microglial fuel metabolism might be associated with microglial reactivity under different pathophysiological conditions and a microglial fuel switch may thus be the underlying cause of hypothalamic dysregulation, which is associated with obesity.
Introduction

Abbreviations: 2-DG, deoxyglucose; ATP, adenosine triphosphate; CNS, central nervous system; GLUT, glucose transporter; BBB, blood brain barrier; LPS, lipopolysaccharide; IFNγ, interferon γ; HK2, hexokinase 2; ICU, intensive care unit; LPL, lipoprotein lipase; ACS, acyl-CoA synthetase; ACSL, acyl-CoA synthetase long chain; ROS, reactive oxygen species; ICV, intracerebroventricular; GPR120, G protein-coupled receptor 120; PUFA, polyunsaturated fatty acid; LPS, Lipopolysaccharide; TLR4, toll-like receptor 4; LCFA, long chain fatty acid; SCFA, short chain fatty acid; FFAR2, free fatty acid receptor 2; GLS, glutaminase; GLUD1, glutamate dehydrogenase 1; SLC1A5, solute carrier family 1 member 5; GABA_R, gamma-aminobutyric acid B receptor; HBMG1, high-mobility group box 1; IL-1β, interleukin-1β; TNF-α tumor necrosis factor-α; GLP-1, glucagon-like peptide-1; CX3CL1, chemokine (C-X3-C motif) ligand 1

1. Introduction

Neurons in the central nervous system require a healthy microenvironment to survive, the maintenance of which critically depends on the innate immune cells, the microglia. Microglia can initiate immune protection under physiological or pathological conditions to efficiently clear away metabolic wastes, cellular debris and pathogens (Hughes 2012). When microglial activity cannot match the demands of the immune defense, the homeostatic and “clean” microenvironment will not be maintained properly (Kreutzberg 1996), and various aspects of neuronal function will be impaired. Under physiological conditions, the debris production in the microenvironment is largely derived from active neurons, especially at the synaptic level (so-called synaptic debris) (Tremblay 2011). Several studies suggest that microglia play an important role in the regulation of synaptic plasticity (Eyo and Dailey 2013, Parkhurst, Yang et al. 2013). Plasticity of synaptic activity is an essential part of neurophysiology in a variety of neural circuits, such as those in the hypothalamus that are involved in the control of energy homeostasis (Yang, Atasoy et al. 2011). Thus properly functioning microglia play an important role in the maintenance of hypothalamic neural circuits.

Microglia are heterogeneously distributed throughout the brain and account for about 10–15% of cells in the brain (Lawson, Perry et al. 1990, Yang, Lin et al. 2013). Microglia function is usually considered to be a “double-edged sword”: under different circumstances microglia can be either beneficial or detrimental to the brain. During development, microglia control the wiring of the brain by regulating processes such as programmed cell death, synaptic pruning and synapse maturation (Bilimoria and Stevens 2015). In the adult brain, microglia maintain a healthy microenvironment by actively surveying the brain parenchyma, scanning for abnormalities and making contact with synapses and neuronal soma (Wake, Moorhouse et al. 2011). As the brains innate immune cells, microglia can phagocytose invading pathogens and clear away debris, thereby restoring homeostasis in the CNS (Mariani and Kielian 2009).
Multiple studies have shown that microglia act as neuroprotectors in a number of ways, including synaptic stripping, promoting neurogenesis and suppressing destructive inflammation by the release of anti-inflammatory cytokines such as IL-10 and TGFβ (Chen and Trapp 2016). But microglia have also been shown to have beneficial effects on the brain. Activation of microglia has been implicated in neurodegenerative diseases such as amyotrophic lateral sclerosis, Huntington disease, multiple sclerosis and Alzheimer’s disease (Cartier, Lewis et al. 2014). Activated microglia are also found in the hypothalamus in diet-induced obesity (Thaler, Yi et al. 2012). Whether activation of microglia is an effect or a cause of this disease-related neurodegeneration, or whether microglia activity actually limit neurodegeneration is currently an area of intense study.

Microglia are highly dynamic cells and the protrusions of so-called “resting” microglia are continuously moving around, scanning the brain (Davalos, Grutzendler et al. 2005, Nimmerjahn, Kirchhoff et al. 2005). After activation, e.g. by damaged neurons or invading microorganisms, microglia rapidly change shape by extending processes towards the damaged site and retracting processes facing the other way (Koizumi, Shigemoto-Mogami et al. 2007). These processes are employed to phagocytize cellular debris and damaged neurons (Fu, Shen et al. 2014). Microglia are also able to replace their scanning processes with new, highly motile processes so they can translocate their complete cell body to an injured site (Stence, Waite et al. 2001). Morphogenesis, phagocytosis and translocation all require dynamic reorganization of the actin cytoskeleton, for which vast amounts of adenosine triphosphate (ATP) are necessary (Engl and Attwell 2015). In order to meet this demand of non-signaling energy, microglia express transporters for the three main energy substrates, indicating a flexible use of available energy resources (Zhang, Chen et al. 2014).

Prolonged hypercaloric challenge results in hypothalamic dysregulation, which coincides with an activation of hypothalamic microglia (Thaler, Yi et al. 2012). After hypercaloric challenge, mediobasal hypothalamic microglia enter a reactive stage, which is initially transient, suggesting a beneficial response. Continued hypercaloric challenge reestablishes the reactive stage of microglia, with possible detrimental effects to surrounding neurons (Thaler, Yi et al. 2012). How a hypercaloric environment is associated with hypothalamic microglial activation is unclear. Saturated fat is supposed to be one of the main triggers, but the driving force behind the uptake of saturated fat into the microglia is unclear, especially regarding the brain fuel dogma, which states that glucose is the main energy resource. So far, very little is known about the hypothalamic microglial energy influx mechanism. This review will summarize microglial energy metabolism in general and discuss the possible links between hypothalamic microglial reactivity and its energy metabolism in metabolic disorders.

2 Microglial energy metabolism

Cellular energy demand determines the energy production and energy substrate influx into
the cell. Under physiological conditions each cell type has a specific metabolic profile for the energy substrates it uses, but changes in the microenvironment or cellular activity can induce a switch of this metabolic profile. For instance, it is known that activation induces peripheral macrophages to switch from oxidative phosphorylation to anaerobic glycolysis, in order to increase ATP production (Kelly and O’Neill 2015). A similar metabolic switch, known as the Warburg effect, has been well-established in specific tumor cells and serves to maintain their increased energy demand (Griffin and Shockcor 2004). Furthermore, it has been shown in a human liver cell line that glutamine deprivation results in upregulation of lipid metabolism (Long, Muhamad et al. 2016). These studies show that changes in energy demand can induce different pathways to metabolize energy substrates, or promote the use of a different substrate. As microglia are in constant need of ATP, particularly after activation, and glucose availability in the brain is not infinite, one would speculate that microglia are capable of a flexible use of energy substrates as well. It is therefore not surprising that microglia show high expression of genes involved in the metabolism of the three major energy substrates. A metabolic switch might be necessary to accommodate the beneficial role of microglia in certain pathophysiological conditions. However, it is also conceivable that a metabolic switch of microglia might be accompanied by the generation of metabolites that alter neuronal health. However, very little is known yet about how microglial cellular metabolism is integrated in their functionality.

2.1 Glucose
Glucose is the main energy source for the brain. The concentration of glucose in the brain is around 20% of that of the glucose concentration in the blood (Dunn-Meynell, Sanders et al. 2009). Five main glucose transporters (GLUT 1-5) mediate glucose uptake in the brain and various peripheral tissues (Thorens and Mueckler 2010). GLUTs 1 and 3 are expressed in many tissues at variable levels and are thought to regulate basal glucose uptake. Once in the brain, glucose can be taken up by neurons with their high affinity glucose transporters 3 (GLUT3), ensuring a constant influx of glucose even when glucose levels are low (Mergenthaler, Lindauer et al. 2013). Similar to neurons, microglia also express GLUT3 (figure 1), but apart from that, microglia can also express other GLUTs under different circumstances. Indeed, previous studies have indicated different GLUTs to be expressed on microglia, in order to ensure sufficient glucose influx to meet the energy demand.

Microglial survival vitally depends on sufficient glucose supply. Culturing primary mice microglia in glucose-free balanced salt solution resulted in 74% cell death within the first 30 hours and even 98% after 48 hours (Yenari and Giffard 2001). Supplementing the balanced salt solution with 5.5 mM glucose decreased microglial death to less than 20% the first 30 hours but a delay in cell death occurred up to 73% after 48 hours (Yenari and Giffard 2001). This suggests that glucose is essential for microglial survival, although survival does not solely depend on glucose in vitro. The effect of glucose deprivation on microglia in vivo is less clear.
Early studies in rats show neurodegeneration with ensuing gliosis one week after a 30 minute period of insulin-induced hypoglycemia (Auer, Kalimo et al. 1985). A more recent study showed significant activation of CD11b positive microglia together with neurodegeneration, one week after 30 minutes of insulin-induced hypoglycemia (Kim, Yoo et al. 2015). In this study, both microglia activation and neurodegeneration could be attenuated by injection of melatonin right after the hypoglycemic period. Melatonin has been shown to impair NADPH oxidase assembly in microglia, thereby effectively decreasing microglial ROS production (Zhou, Zhang et al. 2008). Researchers imply that the degenerative effect of insulin-induced hypoglycemia on neurons is, at least partly, driven by microglia activation. On the other hand, several studies have reported neuroprotective effects when glucose utilization was inhibited using a different method. Treatment with 2-deoxy-D-glucose (2-DG), a glucose analogue blocking glycolysis by inhibiting hexokinase activity, reduces cellular glucose utilization and shows a neuroprotective effect in pathologies such as epilepsy and Parkinson’s disease (Duan and Mattson 1999, Garriga-Canut, Schoenike et al. 2006). Different (non-glial) mechanisms underlying the neuroprotective effect of 2-DG were suggested, but one study showed a reduction of activated microglia in an Alzheimer’s disease mouse model treated with 2-DG (Yao, Chen et al. 2011). In this study the reduction in activated microglia is clear, but whether this is a cause or consequence is unclear. Some in vitro studies show evidence that 2-DG can inhibit microglia, which decreases neurodegeneration. Using neuronal-glial co-cultures, amyloid beta or trauma-induced neuronal loss could be prevented by depleting microglia from culture using 2-DG under normoxic conditions (Vilalta and Brown 2014). 2-DG mainly inhibits anaerobic glycolytic glucose utilization and to a lesser extent oxidative phosphorylation of glucose, which suggests that microglia are more dependent on anaerobic glycolysis than neurons are. Under hypoxic conditions 2-DG treatment not only depleted microglia but also aggravated neuronal loss, probably because during hypoxia neurons rely more on anaerobic glycolysis for ATP production than when oxygen is present (Vilalta and Brown 2014). These studies suggest that microglia can be activated and cause neuronal damage when glucose levels are low. On the other hand, when glycolytic glucose utilization is blocked, neurons do not seem to be affected, but microglia become necrotic, at least in vitro. A similar dependency of glycolytic utilization of glucose is seen in high energy demanding cancer cells, and inhibition of glycolysis has been considered as anti-cancer treatment (Pelicano, Martin et al. 2006). In fact, 2-DG has reached the stage of clinical trials as a combination therapy in the treatment of advanced solid tumors (Raez, Papadopoulos et al. 2013). Whether the 2-DG-induced microglia depletion seen in vitro plays a role in the neuroprotective effects of 2-DG in vivo remains unclear.

The increased energy demand of activated microglia can possibly be met by increasing the glycolytic use of glucose, which is also seen in activated macrophages (Kelly and O’Neill 2015). Similar to macrophages, quiescent microglia are likely to rely on oxidative phosphorylation of glucose for their ATP production. When stimulated, microglia switch from oxidative
phosphorylation to glycolytic metabolism of glucose (Voloboueva, Emery et al. 2013). Activation with Lipopolysaccharide (LPS) not only increased lactate production but also decreased mitochondrial oxygen consumption and mitochondrial ATP production, indicative of increased glycolysis and decreased oxidative phosphorylation (Voloboueva, Emery et al. 2013). Another study confirmed increased lactate production by activated microglia when stimulated with both LPS and IFNγ (Gimeno-Bayon, Lopez-Lopez et al. 2014). This study also showed an upregulation of the glucose transporters GLUT1 and GLUT4 and of hexokinase 2 (HK2) (Gimeno-Bayon, Lopez-Lopez et al. 2014), a key enzyme in glycolysis (figure 1). The glucose transporters were proposed to increase glucose influx, whereas HK2 is thought to be a key promotor of aerobic glycolysis and has been shown to be upregulated in high energy demanding brain tumors (Wolf, Agnihotri et al. 2011). Recent transcriptome studies on both human and mouse brain tissue show high expression of HK2 in microglia (Hickman, Kingery et al. 2013, Lavin, Winter et al. 2014, Zhang, Chen et al. 2014). Together, these studies suggest that microglia adjust their glucose metabolic profile after a change in activity state, possibly to meet an increased energy demand.

Low glucose levels induce microglial death, but high glucose levels have been shown to augment microglial activation. In postmortem brain tissue of critically ill septic patients treated in the ICU, an attenuation of microglial activation was found in patients who had controlled glucose levels before death (Sonneville, den Hertog et al. 2012). Insulin controlled euglycemic patients showed less microglial activity and neuronal damage compared to untreated hyperglycemic patients (Sonneville, den Hertog et al. 2012). In the same study, a hyper-inflammatory response was induced by applying third-degree burn injuries to rabbits. In this setting, uncontrolled hyperglycemic rabbits showed increased microglial activation, which could be prevented by maintaining insulin-induced euglycemia (Sonneville, den Hertog et al. 2012). However, whether this hyperglycemia-induced microglial activation needs inflammatory pre-conditioning is not clear. A number of in vitro studies showed comparable effects of glucose on microglial activation. One study, using primary rat microglia, showed that increasing the glucose concentration from 10 mM to 25 mM resulted in a 4-fold increased secretion of the proinflammatory cytokine TNFα (Quan, Jiang et al. 2011). A second study, using primary rat microglia cultures, showed that TNFα secretion doubled when glucose concentration was raised from 25 mM to 50 mM (Zhang, Dong et al. 2015). In the same study, the presence of low levels of LPS in combination with high levels of glucose (35 mM) showed a dramatic amplification of TNFα secretion by microglia, which was not seen in the 25 mM glucose condition. This indicates that high glucose levels can enhance the LPS-induced inflammatory response in microglia cultures.

Interestingly, microglia appear to be the only cells in the CNS expressing GLUT5 (Horikoshi, Sasaki et al. 2003). The exact function of GLUT5 in microglia in relation to glucose metabolism is not known. What is known is that GLUT5 has a low affinity for glucose, similar to GLUT2 (Manolescu, Witkowska et al. 2007). This could mean that GLUT5 has a function that is similar
to that of GLUT2, e.g. to enable neurons and pancreatic β-cells to sense fluctuating glucose concentrations (Elizondo-Vega, Cortes-Campos et al. 2015, Leloup, Allard et al. 2016). In contrast to the low glucose affinity of GLUT5, its affinity for fructose is much higher. Fructose is a monosaccharide closely related to glucose and a major component of our modern diet. Accumulating evidence from both human and animal studies shows that an excess of fructose causes an increased risk of metabolic disorders such as type II diabetes and insulin resistance, as well as an increased risk of neuroinflammation (Montonen, Jarvinen et al. 2007, Lin, Chan et al. 2016, Xu, Yu et al. 2016). It has been shown that fructose fed rats have activated microglia and that primary rat microglia cultured under high fructose conditions showed upregulation of inflammatory pathways (Li, Ge et al. 2015). Thus, microglia could play a key role in these fructose-induced metabolic disorders, possibly via a hypothalamic mechanism.

Most of the data we summarized about microglial glucose metabolism are from in vitro studies. However, the interpretation of data from in vitro studies needs to be done with caution when they contradict those from in vivo studies. Humans are considered to be diabetic with a fasting plasma glucose concentration above 7 mM or a 2-hour postprandial concentration above 11.1 mM (Giugliano, Ceriello et al. 2008). In diabetic mice, continuous measurements of blood glucose showed a mean concentration of approximately 20 mM, with peak concentrations up to 30 mM. These are the plasma concentrations; brain glucose concentrations are thought to be 5-fold lower than that of plasma (Dunn-Meynell, Sanders et al. 2009). Yet, in vitro studies usually culture microglia under 25 mM glucose conditions (Dai, Li et al. 2015, Zhang, Dong et al. 2015, Beins, Ulas et al. 2016, Vinoth Kumar, Oh et al. 2016), a concentration rarely seen in the brain during physiological or pathophysiological conditions. In leptin receptor mutant db/db mice, which are hyperglycemic, no microglial activation in the hypothalamus was observed (Gao, Ottaway et al. 2014), indicating that brain glucose may indeed be kept at a lower concentration.

### 2.2 Fatty acids

Fatty acids can be categorized into short, medium, long and very long-chain fatty acids and are called saturated when they contain no double bond, and unsaturated when they contain at least one double bond. Once taken up by the digestive system, fatty acids either reach the circulation directly or are released into the lymphatic system as chylomicrons. Peripheral tissue can take up fatty acids by releasing them from the chylomicrons by lipoprotein lipase (LPL) bound to endothelial cells (Niot, Poirier et al. 2009). Once inside the cell, fatty acids can be used by mitochondria to generate ATP through a process called β-oxidation.

In the brain, fatty acids are not generally considered to be a major substrate for ATP production. Nevertheless, fatty acids are taken up by the brain, and some studies report that fatty acid oxidation constitutes 20% of the total brain energy requirement (Ebert, Haller et al. 2003). Additionally, proteins involved in fatty acid metabolism, such as carnitine, are present in the
brain, suggesting that fatty acid metabolism takes place in the brain (Jones, McDonald et al. 2010). There are still two popular theories to explain the transport of fatty acids across the blood brain barrier (BBB), one theory proposes passive diffusion (Mashek and Coleman 2006, Hamilton and Brunaldi 2007) and the other proposes a protein-mediated transport mechanism (Mitchell and Hatch 2011). Cellular uptake of fatty acids appears to be mainly regulated by trapping them inside the cell by their β-oxidation into downstream metabolic intermediates like acyl-CoA. The β-oxidation of fatty acids into acyl-CoA is regulated by acyl-CoA synthetases (ACSSs). Indeed, overexpression of the long-chain family members 4 and 5 of ACS (ACSL4 and ACSL5) in cell cultures, increased fatty acid uptake by 67% and 25–30%, respectively (Heimli, Hollung et al. 2003, Mashek, McKenzie et al. 2006). In the brain, astrocytes have been considered to be the only cells capable of β-oxidizing fatty acids, but very recent transcriptome data show that microglia also express ACSSs such as ACSL1, 3, 4 and 5 (Zhang, Chen et al. 2014) (figure 1). Furthermore, microglia express relatively high levels of LPL (Zhang, Chen et al. 2014) (figure 1), necessary for the release of fatty acids from triglycerides. This could indicate that also microglia are able to β-oxidize fatty acids and use them as an energy source. Utilization of fatty acids as an energy source can, in part, sustain the increased energy demand of activated microglia. Fatty acids have been shown to increase ROS production in a wide variety of cell types, including brain cells (Schonfeld and Wojtczak 2008). The production of ROS is the main contributor to cellular oxidative stress, a pathological feature found in a wide range of neurological diseases (Chen, Guo et al. 2012, Carvalho, Firuzi et al. 2016). Brain tissue is prone to be damaged by ROS due to the relatively low levels of anti-oxidants and its high content of unsaturated lipids (i.e., containing double bonds), which are especially vulnerable to damaging modifications by ROS (Uttara, Singh et al. 2009). In phagocytic neutrophils, fatty acids are known to stimulate ROS production by interfering with the plasma membrane NADPH oxidase system (Schonfeld and Wojtczak 2008). Complex assembly of the two membrane subunits p22phox and gp91phox is regulated by three regulatory cytosolic components p40phox, p47phox and p67phox, and a small GTPase, either Rac1 or Rac2 (Gonzalez, Agapito et al. 2007). Fatty acids are assumed to interfere with these components, resulting in an increased production of ROS (Schonfeld and Wojtczak 2008). Whether fatty acids induce a similar overproduction of ROS by microglia is not known. However, microglia highly express all the components of the NADPH oxidase system (Zhang, Chen et al. 2014), and the NADPH oxidase system has been shown to be the source of LPS-stimulated extracellular superoxide production by microglia (Bordt and Polster 2014). Furthermore, compared to glucose, fatty acid metabolism requires ~15% more oxygen to generate the same amount of ATP (Schönfeld and Reiser 2013). This fatty acid driven increase in oxygen demand is well tolerated by well-oxygenated organs, such as the heart, but could possibly cause problems in the brain, where oxygen concentrations are lower (Schönfeld and Reiser 2013).

Apart from their possible role as an energy substrate for microglia, fatty acids are also thought
to serve as signaling molecules and to influence microglial activity. In general, unsaturated fatty acids are considered to have a beneficial effect on human health, for instance through their anti-inflammatory effects. Intracerebroventricular (ICV) injections of ω-3 linolenic and ω-9 oleic fatty acid significantly reduced hypothalamic inflammation, not only shown by a reduction of inflammatory markers but also by a reduction of F4/80 positive microglia in the arcuate nucleus (Cintra, Ropelle et al. 2012). Both these fatty acids are supposed to activate the unsaturated fatty acid receptor (GPR120) found on hypothalamic neurons (Cintra, Ropelle et al. 2012). However, recent transcriptome studies in human brain showed expression of GPR120 not only in neuronal cells but also in microglia (Zhang, Chen et al. 2014), indicating that these fatty acids might also activate microglia directly. More evidence of this direct effect was provided by a different study, which showed that pre-treatment of primary microglia with polyunsaturated fatty acids (PUFAs) decreased their inflammatory response to the inflammatory stimulators, myelin and IFNγ (Chen, Zhang et al. 2014). A different in vitro study showed that a mixture of ω-3 PUFAs protected against LPS-mediated cytotoxicity in a microglia cell line (BV2) (Corsi, Dongmo et al. 2015). Incubation with PUFAs also inhibited ROS and nitric oxide production by LPS-activated BV2 cells (Corsi, Dongmo et al. 2015). Together these data indicate that unsaturated fatty acids have an inflammatory-inhibiting effect on activated microglia, but whether this is through the GPR120 receptor remains to be resolved.

Unlike unsaturated fatty acids, saturated fatty acids are generally considered as unhealthy fatty acids. Although the inflammatory effect of saturated fatty acids on adipose tissue and macrophages has been well documented and seems to be mediated through the toll-like receptor 4 (TLR4) (Chait and Kim 2010), not much is known about their effect on microglial activation. An in vivo study showed microglial activation in the hypothalamus when mice were given a diet rich in saturated fatty acids (Valdearcos, Robblee et al. 2014). The same study showed a direct effect of saturated fatty acids, especially the long chain fatty acids (LCFA), on cultured mice microglia (Valdearcos, Robblee et al. 2014). A different study investigated the direct effect of both saturated and unsaturated LCFAs on hypothalamic microglia in vivo. Rats were treated with ICV injections containing different LCFAs, such as palmitic, stearic, linoleic, linolenic, arachidic or behenic acids (Milanski, Degasperi et al. 2009). Only the saturated LCFAs - stearic, arachidic and behenic - induced an inflammatory response (Milanski, Degasperi et al. 2009). It turned out that this inflammatory response could be attenuated by ICV administration of TLR4 inhibitors, which suggests a TLR4 mediated response. Microglia might be involved in the saturated fatty acid-induced inflammation, as they are one of the TLR4 expressing cells in the brain (Milanski, Degasperi et al. 2009). This research suggests that saturated fatty acids possibly induce a microglial-induced inflammatory response in the hypothalamus, but more research is needed to clarify the precise mechanism.

A different kind of saturated fatty acids are the short chain fatty acids (SCFAs), exclusively produced by the microbiota in the colon, the main ones being acetate, propionate and butyrate
SFCAs have been shown to have beneficial effects on appetite regulation and energy homeostasis (Byrne, Chambers et al. 2015), and several mechanisms involved in metabolism have been implicated, including the hypothalamic arcuate nucleus (Frost, Sleeth et al. 2014). Here they show that intraperitoneal administered acetate accumulated in the hypothalamus and also resulted in reduced food intake and induction of an anorexigenic neuropeptide expression profile in the hypothalamus (Frost, Sleeth et al. 2014). Whether microglia play a role in any of these processes that underlie the beneficial effect of SFCAs is not known. However, SCFAs do seem to be of critical importance for microglia functioning in general. Studies using germ free mice, which do not have SCFAs because they lack a microbiota, showed impaired maturation and function of microglia (Erny, Hrabe de Angelis et al. 2015). Microglial impairment could be restored not only by the introduction of a complex microbiota, but also by supplementing SCFAs via the drinking water (Erny, Hrabe de Angelis et al. 2015). A similar microglial impairment was found in SCFA receptor knockout mice (FFAR2−/−), although thus far in mice no FFAR2 expression has been found in microglia or any other cells of the CNS. Instead, expression was only found on peripheral myeloid cells (Erny, Hrabe de Angelis et al. 2015). This suggests that SCFAs indirectly alter microglia functioning (Erny, Hrabe de Angelis et al. 2015). A recent transcriptome study corroborated the lack of FFAR2 expression in the murine CNS, but did find FFAR2 expression in human microglia (Zhang, Chen et al. 2014). The FFAR2 expression in human microglia could be an interspecies difference, but expression is very low so whether this has any relevance is uncertain. Another study showed that the SCFA butyrate protected against an LPS-induced inflammatory response in rat primary microglia, hippocampal slice and co-cultured microglia, astrocytes and cerebellar granule neurons (Huuskonen, Suuronen et al. 2004). In the same study, the opposite effect of SFCAs was seen on a cell line of immortalized microglia (N9), where an enhanced LPS response was found (Huuskonen, Suuronen et al. 2004). The majority of these studies thus suggest that SCFAs can influence microglial functioning in an anti-inflammatory fashion, but the role of the FFAR2 receptor remains unclear. Whether the anti-inflammatory effect of SFCAs on microglia found in-vitro possibly plays a role in the beneficial effect on appetite regulation in vivo might be interesting avenue of investigation.

2.3 Glutamine

Glutamine is the most abundant amino acid in plasma and has been considered to be unable to freely pass the BBB. Multiple glutamine transporters are present in the BBB and these are mainly responsible for moving glutamine from the brain into the circulation. However, some might also play a role in glutamine uptake into the brain (Xiang, Ennis et al. 2003). In the brain, astrocytes produce glutamine from excess glutamate and ammonia in order to protect neurons against excitotoxicity (Suarez, Bodega et al. 2002). Both glutamate and ammonia have been shown to be neurotoxic (Manev, Favaron et al. 1989, Felipo and Butterworth 2002, Suarez,
Bodega et al. 2002), and subcutaneous glutamate injections resulted in an obese phenotype (Olney 1969).

Although glutamine is, first and foremost, the end product of glutamate and ammonia metabolism, it can also be used as an energy source in the brain. Cellular uptake of glutamine is accomplished by several amino acid transporters (Pochini, Scalise et al. 2014). The amino acid transporter mainly responsible for glutamine uptake is solute linked carrier family 1 member A5 (SLC1A5), which has a high affinity for glutamine and has been shown to be upregulated in several high-energy demanding tumors (De Vitto, Perez-Valencia et al. 2016). When glutamine is taken up it can be broken down to α-ketoglutarate via glutaminase (GLS) and glutamate dehydrogenase (GLUD1). GLS, GLUD1 as well as SLC1A5 are highly expressed in human and mouse microglia (figure 1). The majority of amino acid transporters listed in (Pochini, Scalise et al. 2014) are also expressed in human and mouse microglia (figure 1), indicating microglia have an active glutamine metabolism pathway.

Several studies have shown that microglial glutamate production is neurotoxic. A mouse model with upregulated microglial levels of the glutamine transporter SLC38A1 (figure 1) constitutively produced and excreted glutamate into the interstitial space, which resulted in low levels of neurotoxicity (Jin, Horiuchi et al. 2015). Research using primary cultures of human microglia showed that infection with HIV-1 increased the extracellular glutamate levels and amplified neurotoxicity (Huang, Zhao et al. 2011). Cultured microglia infected with Japanese encephalitis virus showed elevated release of glutamate into the medium, and this conditioned medium could thus provoke significant neuronal damage in neuronal cultures (Chen, Ou et al. 2012).

The role of hypothalamic microglial glutamine metabolism and its interactions with other energy resources is still completely unknown. More studies are needed to clarify the degree to which microglia depend on glucose, fatty acids and glutamine for their energy requirements.

2.4 Microglia, astrocyte and neuron interaction

Microglial energy consumption is dependent on the degree of activity of the microglia. Healthy neurons and astrocytes keep them in the relatively low energy demanding “resting state” (Shih, Fernandes et al. 2006, Eyo and Wu 2013). Detection of neuronal activity is important for microglia, indicated by the great variety of neurotransmitter receptors they express, including glutamate, GABA$\text{B}$R, dopamine, purinergic, cholinergic and opioid receptors (Pocock and Kettenmann 2007). Possibly, the amount of extra synaptic neurotransmitters released during normal brain function can suppress microglial activation (Pocock and Kettenmann 2007).

Astrocyte-conditioned medium has been shown to keep microglia in a quiescent state (Shih, Fernandes et al. 2006). Activation of both microglia and astrocytes has been associated with various neurodegenerative diseases (Mandybur, Beach, Walker et al. 1989, Bates, Fonte et al. 2002, Alfonso-Loeches, Pascual-Lucas et al. 2010, Howell, Rundle et al. 2010, Popescu,
Pirko et al. 2013). Whether astrocytes can activate microglia or if microglia are activated by astrocytes first, has been a long-standing debate (Liu, Tang et al. 2011). One study has shown that cultured astrocytes could be activated with LPS, indicating astrocytes can be activated without microglia (Brahmachari, Fung et al. 2006). However, astrocyte cultures frequently contain small amounts of microglia, which renders interpretation of these results difficult. Research using astrocyte cultures with depleted residual microglia showed a decreased LPS response, but when these astrocyte cultures were reconstituted with microglia, the LPS response returned, and even exceeded the LPS response of microglia alone (Barbierato, Facci et al. 2013). Furthermore, research focusing on high-mobility group box 1 (HBMG1)-induced edema, showed that astrocyte cultures only showed a response to HBMG1 when microglia were present (Ohnishi, Monda et al. 2014). A different study showed that the LPS-induced astrocyte activation needed functional receptors for IL-1β and TNF-α, the ligands of which are produced by microglia (Abudara, Roux et al. 2015). Together these data show that astrocytes can interact with microglia and keep them in a quiescent state. Whether astrocytes can also activate microglia remains obscure.

2.5 Microglial reactivity and hypothalamic dysfunction in control of energy homeostasis

In diet-induced obese mice, activated microglia are found specifically in the mediobasal hypothalamus (Thaler, Yi et al. 2012). It is not surprising that microglia in the mediobasal hypothalamus are activated by certain blood borne factors, because this part of the brain is in close proximity to the median eminence, a region known for having a locally decreased blood-brain barrier (BBB) function (Johnson and Gross 1993). Because of this, circulating fatty acids and other blood borne factors can diffuse more directly into this region (Jastroch, Morin et al. 2014). Hypothalamic inflammation in this part of the brain has been associated with metabolic disorders (Thaler, Yi et al. 2012, Dorfman and Thaler 2015). Two key questions raised by the association of hypothalamic microglial activation and metabolic disorders are: 1) are there fuel switches between fatty acids, glucose and/or glutamine in activated microglia and 2) which stimulus is linked to which microglial fuel preference and is thus responsible for the different microglial metabolic phenotypes? Clarifying microglial fuel preference might prove to be vital for understanding microglial pathogenesis and might become of interest as a cell-specific therapeutic strategy to normalize microglial function.

2.6 Therapeutic strategy targeting microglia in obesity treatment

Most anti-obesity treatments target the neurons that are responsible for the regulation of energy metabolism by either inhibiting the orexigenic neurons or stimulating the anorexigenic neurons. Due to a lack of specificity, anti-obesity drugs targeting these regulatory neurons either show little weight reduction or have serious side effects, as exemplified by the drugs targeting neuropeptide Y, serotonin and endocannabinoids (Di Marzo 2008, Sargent and Moore
New targets are needed for the treatment of obesity and these might be found in microglia, in view of their newly identified roles in hypothalamic dysregulation associated with obesity. In DIO mice, depletion of microglia in the mediobasal hypothalamus abolished diet-induced inflammation and reduced food intake (Valdearcos, Robblee et al. 2014). Treatment of microglia-neuron co-cultures with 2-DG has shown that inhibition specifically of microglia increases neuronal survival (Vilalta and Brown 2014). Anti-inflammatory drugs like corticosteroid analogues would be a possible treatment to dampen the microglial inflammation during obesity in humans, but these drugs have been shown to have serious side effects, such as osteoporosis, aseptic joint necrosis and adrenal insufficiency (Buchman 2001). A more specific effect, i.e., only inhibiting microglia, is desired when using drugs like corticosteroids. A new and promising strategy seems to be the cell specific pharmacotherapy as introduced by Dimarchi & Tschöp (Finan, Yang et al. 2012). They successfully targeted specific tissues, while at the same time preventing adverse effects in other tissues, by conjugating estrogen to glucagon-like peptide-1 (GLP-1). Only cells expressing both estrogen and GLP-1 receptors were affected by this conjugate (Finan, Yang et al. 2012). A similar strategy could be used to specifically inhibit microglial activation during obesity. In the CNS, the receptor for chemokine (C-X3-C motif) ligand 1 (CX3CL1) is exclusively expressed by microglia. Conjugation of a corticosteroid analogue to CX3CL1 would only target cells expressing the CX3CL1 receptor, without affecting other cells in the CNS (Kalin, Heppner et al. 2015).

3. Conclusion
An increased energy demand by activated microglia may be accompanied by a change in their metabolic profile. A switch towards increased fatty acids and glutamine metabolism might result in the generation of metabolites, which are harmful for neuronal survival. Furthermore, different fatty acids have been shown to either have proinflammatory or anti-inflammatory effects on microglia. As the hypothalamus is in close contact with the circulation, this region is specifically prone to be influenced by a change in circulating blood borne factors. Also, obesity has been associated with hypothalamic inflammation, possibly caused by activated microglia. If activated microglia create an unhealthy microenvironment for neuronal survival, and cause hypothalamic dysfunction, eventually leading to obesity needs to be answered. Research into the cellular metabolism of how activated microglia meet their energy demands and which blood borne factors can induce such an activation might shed some light onto the underlying causes of obesity.

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Figure 1. Overview of metabolic pathways for the three major energy substrates in microglia. Glucose can enter microglia via glucose transporters GLUT1, GLUT3, GLUT4 and GLUT5. GLUT5 has also been shown to facilitate the passage of fructose across the plasma membrane. Once inside the cell, glucose undergoes glycolysis which, in part, is mediated by hexokinase2 (HK2). The generated pyruvate can either enter the mitochondria where it is further metabolized with the use of oxygen by the tricarboxylic acid (TCA) and electron transport chain (ETC), or pyruvate can be further metabolized in the cytosol without the use of oxygen, resulting in the formation and secretion of lactate. 2-Deoxy-D-glucose (2-DG), a glucose analogue, uses the glucose transporters to enter the cell where it inhibits glycolysis. Lipoprotein lipase (LPL) mediates the cellular uptake of fatty acids. Long chain fatty acyl CoA synthetase (ACSL) catalyzes the formation of fatty acyl CoA, which can only enter the mitochondria together with the carrier protein carnitine. Once inside the mitochondria, fatty acyl CoA is β-oxidized into acetyl-CoA, which enters the TCA cycle and subsequently the ETC in order to generate ATP. GPR120 is known to bind unsaturated fatty acids and is possibly involved in the anti-inflammatory effects of unsaturated fatty acids. Microglia highly express all the components of the NAPDH oxidase complex, which has been shown, in macrophages, to be stimulated by fatty acids to increase its production of reactive oxygen species (ROS). The glutamine receptors SLC1A5 and SLC38A1 are expressed with microglia and enable microglia to take up glutamine. Inside the mitochondria glutamine is converted to glutamate and ammonia (NH₄⁺) by the enzyme glutaminase (GLS), glutamate is further metabolized by the enzyme glutamate dehydrogenase 1 (GLUD1) to α-ketoglutarate, which can enter the TCA cycle. Both microglial-produced glutamate and ammonia have been shown to have neurotoxic effects.
References


“n-3 PUFA supplementation benefits microglial responses to myelin pathology.” Scientific Reports 4: 7458.


Diet-induced obesity disturbs microglial immunometabolism in a time-of-day manner

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Abstract:
Background: Disturbance of immunometabolic signaling is a key process involved in the progression of obesity. Microglial cells – the resident immune cells in the brain, initiate local immune responses. It is known that hypercaloric diets lead to microglial activation. Previously, we observed that hypothalamic microglial cells from mice fed high-fat diet (HFD) lose their day/night rhythm and are constantly activated. However, little is known about daily rhythmicity in microglial circadian, immune and metabolic functions, either in lean or obese conditions. Therefore, we hypothesized that HFD disturbs microglial immunometabolism in a day/night-dependent manner.

Methods: Obesity was induced in Wistar rats by feeding them a HFD ad libitum for the duration of eight weeks. Microglia were isolated from HFD- and chow-fed control animals at six time points during 24h (every four hours starting 2h after lights on, i.e. Zeitgeber Time 2 (ZT2)). Gene expression was evaluated using quantitative RT-PCR. JTK_Cycle software was used to estimate daily rhythmicity. Statistical analysis was performed with two-way ANOVA test.

Results: Consumption of the obesogenic diet resulted in a 40 g significantly higher body weight gain in week eight, compared to chow diet (p<0.0001), in association with increased adiposity. We observed significant rhythmicity of circadian clock genes in microglia under chow conditions, which was partially lost in diet-induced obesity (DIO). Microglial immune gene expression also showed time-of-day differences, which were disrupted in HFD-fed animals. Microglia responded to the obesogenic conditions by a shift of substrate utilization with a decrease of glutamate and glucose metabolism in the active period of the animals, and an overall increase of lipid metabolism, as indicated by gene expression evaluation. Additionally, data on mitochondria bioenergetics and dynamics suggested an increased energy production in microglia during the inactive period on HFD. Finally, evaluation of monocyte functional gene expression showed small or absent effect of HFD on peripheral myeloid cells, suggesting a cell-specific microglial inflammatory response in DIO.

Conclusions: An obesogenic diet affects microglial immunometabolism in a time-of-day dependent manner. Given the central role of the brain in energy metabolism, a better knowledge of daily rhythms in microglial immunometabolism could lead to a better understanding of the pathogenesis of obesity.
**Introduction:**

Arising evidence highlights the disturbed interaction between immunity and metabolism as a key player in the pathogenesis of obesity (Mathis and Shoelson 2011, Hotamisligil 2017, Lee, Wollam et al. 2018). Immune cell function is highly dependent on metabolic adaptation of the immune cells, allowing for abrupt shifts in energy utilization, thus promoting either a resting or an activated state (Norata, Caligiuri et al. 2015). Moreover, distinct immune cell populations show specific metabolic patterns, modulating their functional properties (Norata, Caligiuri et al. 2015). In the brain, microglia are involved in maintaining brain homeostasis by surveying the environment, sensing invading pathogens and phagocyting dead neurons and cellular debris, thus eliciting an innate immune response (Mariani and Kielian 2009, Yang, Han et al. 2010). Microglial metabolic reprogramming is associated with polarization to pro- or anti-inflammatory state, which involves both functional and phenotypic plasticity (Orihuela, McPherson et al. 2016, Butovsky and Weiner 2018). It has been shown that hypercaloric environment induces a proinflammatory response in the hypothalamus via NF-kB and toll-like receptor activation, leading to disturbed energy homeostasis (De Souza, Araujo et al. 2005, Morari, Anhe et al. 2014). This could be due to hypothalamic microglial activation as seen in rodents fed an obesogenic diet (Gao, Ottaway et al. 2014, Valdearcos, Robblee et al. 2014, Baufeld, Osterloh et al. 2016, Gao, Bielohuby et al. 2017). We observed that under physiological conditions in mice, microglial cells exert their function in a strict time-of-day manner with higher activity during the dark, active phase, compared to the light, sleep phase (Yi, Walter et al. 2017). However, this day-night rhythm was abolished in animals fed an obesogenic, high-fat diet (HFD), suggesting an interaction of diet content and daily rhythms. Indeed, recent evidence suggest an involvement of circadian function in the progression of obesity (Froy 2010, Sun, Feng et al. 2018). It is well known now that a master circadian clock in mammals generates daily rhythms in behavioral, physiological and hormonal processes to allow adaptation to daily environmental changes, thus optimizing metabolic function to the time of day (Kalsbeek, Palm et al. 2006). However, little is known about daily rhythms in microglial function. Therefore, we performed a detailed investigation of daily rhythmicity in microglial immunometabolism in lean and obese rats. As mentioned earlier, many studies have focused on hypothalamic microglial inflammatory response due to the clear relation between the hypothalamus and energy homeostasis. Here, we chose to evaluate cortical microglial activation, to expand on available knowledge on microglial immunometabolism in obesity outside of the hypothalamus.

We induced obesity with HFD for the duration of 8 weeks in rats and evaluated the expression of key clock genes involved in maintaining circadian rhythms (Fig.1). Microglial cells, as many other immune cells, have a high metabolic demand (Olenchock, Rathmell et al. 2017). Therefore, we also evaluated the expression of key genes involved in microglial glucose, lipid and glutamate metabolism. As higher activity and substrate utilization require higher
energy production we also assessed the state of mitochondria bioenergetics and dynamics in response to either healthy or obesogenic diet. The immune state of the cells was studied by evaluating cytokine production and phagocytosis (Fig. 1). Our results showed time-of-day disturbances in microglial circadian and inflammatory functions in the obesogenic conditions, accompanied with changes in substrate utilization and energy production. We compared these data to monocytes, isolated from the same animals, to evaluate the state of peripheral myeloid cells in a hypercaloric environment. We observed a small effect of HFD on monocyte function, suggesting a microglia-specific response to hypercaloric intake. These results shed further light on microglial time-of-day innate immunometabolism in health and obesity.

![Figure 1. Microglial circadian, immune, and metabolic profile. Schematic representation of the pathways and/or functions tested on microglial cells from chow- or HFD-fed rats.](image)

**Methods**

**Animals**

Seventy-two male Wistar rats (Charles River, Germany) were group housed on a 12-hour-light/12-hour-dark cycle (lights on at 7:00 am; Zeitgeber time zero (ZT0)) at 22±2°C with access to food and water *ad libitum*. Obesity was induced for the duration of eight weeks, with a diet containing 60 kcal% fat and 20 kcal% carbohydrates (HFD, 5.24 kcal/g, D12492, Research Diets inc.). Control animals were fed a standard chow diet (3.1 kcal/g, 2018, Teklad diets, Invigo). Body weight was monitored once per week, and food intake twice per week.
All studies were approved by the Animal Ethics Committee of the Royal Dutch Academy of Arts and Sciences (KNAW, Amsterdam) and performed according to the guidelines on animal experimentation of the Netherlands Institute for Neuroscience (NIN, Amsterdam).

**Microglia/monocyte isolation and plasma collection**

Animals were sacrificed at six time points during 24h (every four hours starting at ZT2) by euthanasia with 60% CO$_2$/40% O$_2$, followed by decapitation. Perirenal white adipose tissue (pWAT) was dissected for evaluation of fat mass gain, evaluating the amount of pWAT in grams weight. Microglial cells from cerebral cortex were isolated for gene expression analysis using the Percoll isopycnic isolation, as it provides a high cell number (Nikodemova and Watters 2012). Briefly, brains were mechanically homogenized with RPMI 1640 medium (Ref.: 11875-093, Gibco™) and filtered through 70 µm cell strainer (Ref.: 431751, Corning®) in a 15 mL Falcon tube. Brain homogenate was centrifuged for 5’ (380g, 4°C). Pellets were resuspended with 7 mL RPMI medium and mixed with 100% Percoll solution (for 10 mL: 9 mL Percoll® stock (Ref.: 17-5445-01, GE Healthcare, Sigma-Aldrich®) with 1 mL 10x HBSS (Ref.: 14185-045, Gibco™)). The cell suspension was layered slowly on 70% Percoll solution (for 10 mL: 7 mL 100% Percoll solution with 3 mL 1x HBSS (Ref.: 14175-053, Gibco™)) and centrifuged for 30’ (500g, 18°C, break 1/0). Cell debris on the surface was discarded and fuse interphase, containing microglial cells were collected in 8 mL 1x HBSS, followed by centrifuging for 7’ (500g, 18°C, break 9/9). Supernatant was discarded and the microglial cell pellet was used directly for RNA extraction.

During decapitation trunk blood was collected for measurement of different parameters. Briefly, blood was collected in 50 mL Falcon tubes, containing 0.5 M EDTA (ethylenediaminetetraacetic acid). Blood was filtered through a 70 µm cell strainer in a 15 mL Falcon tube and separated for monocyte isolation. For plasma collection, 2 mL blood was centrifuged for 15’ (4000rpm, 4°C, break 9/9). Plasma was collected in a new tube and stored at -80°C until usage. For monocyte isolation, 30 mL lysis buffer (containing 1x ACK; 155 mM NH$_4$Cl; 10 mM KHCO$_3$; 0.1 mM EDTA) was added to approximately 3 mL blood and vortexed gently, followed by incubation at RT for 10-15’. The cell suspension was centrifuged for 5’ (200g, RT, 9/9 break), supernatant was discarded and cells were resuspended in 2 mL PBS-FBS (PBS containing 1% FBS). The new cell suspension was again centrifuged for 5’ (200g, RT, 9/9 break), supernatant was discarded and cells were resuspended in 0.5 mL PBS-FBS. The cell suspension was added to 4.5 mL RPMI medium and layered slowly on 5 mL Ficoll® (Ref.: 17-1440-02, GE Healthcare, Sigma-Aldrich®), followed by centrifuging for 30’ (400g, 20°C, break 1/1). The fuse interphase, containing monocytes, was collected in 8 mL 1x HBSS, followed by centrifuging for 5’ (200g, RT). Supernatant was discarded and the monocyte pellet was used for RNA extraction.
Real-time PCR

For gene expression analysis, RNA from microglial cells and monocytes was extracted using the RNeasy Micro Kit (Cat No. 74004, Qiagen®) according to the manufacturer’s guidelines. RNA was quantified by spectrophotometry at 260 nm (DS 11; Denovix). RNA was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (04897030001; Roche) according to the manufacturer’s guidelines. Levels of mRNA for *Tnfa, Bmal1, Per1, Per2, Cry1, Cry2, Dbp, Reverba, Clock, Gls, Gdh, Gpx1, Csd36, Fas, Lpl1, Opa1, Mfn2, Fis1, Drp1, Pdk4, Ppard, Ikbbk, Cd68, Il1b, Cox4, Atp5b, Atp5g, Hk2, Glut5, Myd88, Sirt1, Hprt* (internal control) and *bactin* (internal control) were measured by semiquantitative real-time PCR on a LightCycler LC480 (Roche), using the SensiFAST SYBR® No-ROX Kit (BIO-98020, GC-Biotech) according to the manufacturer’s guidelines. Expression levels of all genes were normalized to the geometric mean of the internal controls. Primer sequences (see Supplementary Table S1) were designed using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI). Primers were purchased from Sigma-Aldrich® and validated by melt curve analysis and DNA band size and/or purity on agarose gel electrophoresis (data not shown).

Glucose, insulin & non-esterified fatty acids (NEFA) measurements in plasma

Plasma glucose concentrations were measured using the Glucose GOD-PAP kit (Ref. 80009, Biolabo S.A.S.), following the manufacturer’s guidelines. Absorbance of colored samples, proportional to glucose concentration, was measured at 500 nm with Varioskan® Flash spectral scanning multimode reader (Version 40053; Thermo Scientific). Insulin concentrations were measured using Rat Insulin Radioimmunoassay (RIA) Kit (RI-13K; Millipore, Merck), according to the manufacturer’s guidelines. Non-esterified fatty acids concentration in plasma was measured using the NEFA HR(2) reagents (R1 set, Ref. 434-91795; R2 set, Ref. 436-91995; Standard, Ref. 270-77000, Wako Chemicals GmbH) following the adjusted protocol from the Mouse Metabolic Phenotyping Centers [https://www.mmpc.org/shared/document.aspx?id=196&docType=Protocol]. Within-run variations for all measurements fall in the range suggested by the manufacturers.

Statistical analyses

All results are expressed as mean ± SEM. Statistical analyses were performed using GraphPad PRISM (version 7.03, GraphPad Software, Inc.) and JTK_Cycle software (Hughes, Hogenesch et al. 2010). Two-way ANOVA analysis was used for effects of *Diet, Time (ZT)* and *Interaction*. Unpaired t-test’s were used to evaluate the effect of diet for each time point, unless stated otherwise. Sidak’s multiple comparison test was used to compare the effect of diet for the food intake, body weight gain and plasma measurements data (Fig. 2A, B, E-F). One-way ANOVA analysis was used to assess the effect of *Time* for the chow and HFD groups separately. JTK_Cycle analysis p-values were obtained by fitting the data on a curve with fixed
24h period. Results were considered statistically significant when p<0.05.

Results

**HFD intake induces obesity in rats**

We observed that chronic feeding with HFD for eight weeks induced obesogenic phenotype in adult male rats, compared to control animals on the standard chow diet. The HFD rats had a higher caloric intake (Fig. 2A) and a 40g higher body weight gain after eight weeks as compared to controls (Fig. 2B). Moreover, there was a two-fold increase in perirenal white adipose tissue (pWAT) mass in HFD-fed animals compared to controls (Fig. 2C). These results were in line with other literature available on diet-induced obesity (DIO) in rodents (Bahceci, Tuzcu et al. 1999, Gao, Bielohuby et al. 2017). To assess glycemic status at the time of death, we evaluated glucose and insulin concentrations in plasma over the 24h cycle. Control animals showed the expected daily rhythm in glucose concentrations in the plasma (La Fleur, Kalsbeek et al. 1999). However, HFD-fed animals showed increased glucose concentrations during the light phase at ZT6 (inactive period) (Fig. 2D). The overall high levels of glucose concentration in both conditions could be explained by our choice of euthanasia (60% CO$_2$/40% O$_2$), as it has been shown previously that CO$_2$ causes acidosis which stimulates enzymes of the glycolytic pathway, leading to decreased liver glycogen stores and increased plasma glucose concentrations, both in fed and fasted animals (Artwohl, Brown et al. 2006, Zardooz, Rostamkhani et al. 2010). Insulin concentrations were significantly elevated in HFD-fed animals during the dark phase (active period) at ZT18, which could indicate an impaired insulin sensitivity, as glucose concentrations during this period were not elevated, but overall maintained during 24h (Fig. 2E). A similar trend of increased insulin secretion during the dark phase has also been observed in mice on a HFD (Kohsaka, Laposky et al. 2007). Evaluation of the non-esterified fatty acids (NEFA) concentrations in plasma showed a significant increase in HFD-fed animals during the light phase (ZT2-ZT10) compared to chow controls (Fig. 2F). Together, these data indicate metabolic changes towards obesity in animals fed HFD.

**HFD disturbs microglial circadian gene expression**

It has been shown previously that microglial cells express clock genes (Nakazato, Takarada et al. 2011, Fonken, Frank et al. 2015). Diets rich in fat and/or sugar are known to alter circadian rhythms of clock gene expression in peripheral tissue (Pendergast, Branecky et al. 2013, Blancas-Velazquez, Unmehopa et al. 2018). To test whether HFD also disturbs daily microglial rhythmicity, we studied expression of genes within the transcriptional feedback loop – circadian locomotor output cycles kaput (*Clock*) and brain and muscle ARNT-Like 1 (*Bmal1*) - the so-called activators and the repressors – period and cryptochrome genes (*Per1, Per2, Cry1* and *Cry2*). Additionally, we assessed the expression of two other clock genes - reverse viral erythroblastosis oncogene product alfa (*Revberba*), a *Clock* and *Bmal1* repressor,
and D-box binding protein (Dbp), a regulator of peripheral circadian input (Takahashi 2016).

Figure 2. HFD intake leads to obesity in rats. HFD (orange) leads to an increase in food intake (A) ($p_{int}$ <0.0001, $p_{time}$ <0.0001, $p_{diet}$ <0.0001), body weight (B) ($p_{int}$ <0.0001, $p_{time}$ <0.0001, $p_{diet}$ = 0.0003), and fat mass gain, seen as 2 fold increase in perirenal white adipose tissue (C) in rats, compared to chow-fed controls (black). Plasma measurements in non-fasted HFD-fed animals show an increase in glucose at ZT6 (D) ($p_{int}$ = 0.0032, $p_{time}$ = 0.0003, $p_{diet}$ = 0.0874), increased insulin at ZT18 (E) ($p_{int}$ = 0.2802, $p_{time}$ = 0.0042, $p_{diet}$ = 0.0006), and significant increase in non-esterified fatty acids during the light phase (F) ($p_{int}$ <0.0001, $p_{time}$ = 0.0006, $p_{diet}$ <0.0001). Data are presented as means ± SEM. Statistical significance was determined using Two-way ANOVA (A,B) or unpaired t-test (C). Effect of diet within each time point was evaluated with Sidak’s multiple comparison test (A,B,D-F) ($p$<0.05*, $p$<0.01**, $p$<0.001***, $p$<0.0001****).

Control animals fed chow diet showed a clear rhythmic expression for all genes, except Clock and Cry2 (see Supplementary Table S2). Rhythmicity of Bmal1, DBP and Reverba was not influenced by HFD, although a reduced amplitude was observed for DBP and Reverba. There was a gain of rhythm for Clock expression. However, Per1, Per2 and Cry1 showed a loss of rhythmic expression during HFD, as evaluated with JTK_CYCLE (see Supplementary Table S2). Moreover, all genes showed a significant Interaction effect, as well as difference between HFD and chow-fed animals at the transition period between dark and light phase (ZT22 and/or ZT2), (Fig. 3A-H, Table 1). These data point to a clock disturbance, which could lead to irregularity in the expression of other key microglial genes, as it is known that clock genes regulate the expression of 10-20% of all cell genes (Takahashi 2016).
Microglial circadian clock system in HFD-fed and control animals.

Relative gene expression of circadian genes *Bmal1* (A), *Clock* (B), *Cry1* (C), *Cry2* (D), *Per1* (E), *Per2* (F), *Reverba* (G) and *Dbp* (H) in HFD-fed rats (red) compared to Chow-fed controls (black). Data are presented as means ± SEM. Statistical significance was determined using Two-way ANOVA effects for Interaction, Diet and Time (ZT); Student t-test is used for diet effect within a separate time point (p<0.05 *; p<0.01**). Scale (bottom right) represents light (ZT0-12) and dark (ZT12-24) phase.

**Figure 3. Microglial circadian clock system in HFD-fed and control animals.** Relative gene expression of circadian genes *Bmal1* (A), *Clock* (B), *Cry1* (C), *Cry2* (D), *Per1* (E), *Per2* (F), *Reverba* (G) and *Dbp* (H) in HFD-fed rats (red) compared to Chow-fed controls (black). Data are presented as means ± SEM. Statistical significance was determined using Two-way ANOVA effects for Interaction, Diet and Time (ZT); Student t-test is used for diet effect within a separate time point (p<0.05 *; p<0.01**). Scale (bottom right) represents light (ZT0-12) and dark (ZT12-24) phase.

**Microglial time-of-day disturbance of inflammatory signaling during HFD.**

To evaluate the effect of HFD on daily changes in microglial activation, we assessed the relative gene expression of the main cytokines secreted by microglia - tumor necrosis factor α (*Tnfa*) and interleukin 1β (*Il1b*). We observed an increased expression of *Tnfa* at the transition between dark and light phase, as well as increased *Il1b* production at the end of the light period for animals fed HFD, pointing to an increased microglial activation in the obesogenic group, compared to controls (Fig. 4A&B). However, myeloid differentiation primary response 88 (*Myd88*) gene expression, an adaptor for inflammatory signaling pathways, located downstream of *Il1b*, showed a decrease at ZT2 in HFD-fed animals (Fig. 4C). Therefore, we assessed the expression of inhibitor of nuclear factor kappa B kinase subunit beta (*Ikbkb*) as the protein it encodes phosphorylates the inhibitor in the inhibitor/NFkB complex, leading to activation of nuclear factor kappa-light-chain-enhancer of activated B cells NFkB - a transcriptional activator of key genes involved in cell survival, proliferation and inflammatory response. We observed an inverted daily pattern of *Ikbkb* expression between chow and HFD animals, with higher expression at the beginning of the light phase, but lower expression at the end of the dark phase for HFD-fed animals, compared to chow diet controls (Fig. 4D).
Figure 4. Microglial inflammatory signaling is disturbed in DIO. Relative gene expression of innate immunity genes *Tnfa* (A), *Il1b* (B), *Myd88* (C), *Ikbkb* (D) as well as phagocytic indicator gene *Cd68* (E) and *Sirt1* (F) in HFD-fed rats (red) compared to Chow-fed controls (black) evaluated at six time points, starting at ZT2. Data are presented as means ± SEM. Statistical significance was determined using Two-way ANOVA effect for Interaction, Diet and Time (ZT); Student t-test is used for diet effect within a separate time point (p<0.05*; p<0.01**; p<0.001***). Scale (bottom right) represents light (ZT0-12) and dark (ZT12-24) phase.

We also studied gene parameters reflecting the phagocytic capacity of microglia as this is a key function of their immune response in health, as well as different pathologies (Wolf, Boddeke et al. 2017). We evaluated the gene expression of cluster of differentiation 68 (*Cd68*), which encodes for a microglial lysosomal protein, and is a good indicator of phagocytic activity (Zotova, Bharambe et al. 2013). Our results showed an overall steady expression of *Cd68* during the day-night cycle for HFD-fed animals, with a loss of the time-of-day differences, as observed in control animals (Fig. 4E). One-Way ANOVA evaluation of the effect of Time for each group showed a loss of significance during HFD (see Supplementary Table S3). Recent studies have shown that Sirtuin 1 (*Sirt1*) deficiency in microglia is associated with increased *Il1b* production (Cho, Chen et al. 2015). We observed an inverted pattern of expression of *Sirt1* expression in animals fed HFD, compared to controls. Moreover, the significantly lower *Sirt1* expression at ZT10 coincided with an increased expression of *Il1b* at the same time point (Fig. 4F). No significant daily rhythmicity was observed for any of the genes, apart from *Myd88* in Chow-fed animals and *Ikbkb* in HFD-fed animals (see Supplementary Table S2). These data demonstrate that microglial innate immunity is affected in HFD-fed animals, suggesting a disruptive effect of obesogenic diets on the microglial inflammatory response.
**Microglial glutamate metabolism decreases during the dark phase during HFD.**

Glutamate metabolism is a key component in the biosynthesis of nucleic acids and proteins (Schousboe, Scafidi et al. 2014, Yelamanchi, Jayaram et al. 2016). Microglial cells have been shown to be involved in glutamate uptake under physiological conditions, which can be directly converted to glutathione as a defense response against oxidative stress (Persson, Sandberg et al. 2006). This mechanism has also been observed under pathological conditions, where it has been shown that microglial cells express glutamate transporters (Persson and Ronnback 2012). We wanted to assess the state of glutamate substrate utilization in microglial cells under control and obesogenic conditions. We observed that glutaminase (Gls) - a key enzyme in the glutamate pathway that converts glutamine to glutamate, showed an effect of Time in control animals, which was lost during HFD, with a decrease in expression during the dark phase (ZT18) (Fig. 5A) (see Supplementary Table S3). Similar observations were made for glutamate dehydrogenase 1 (Gdh1), a mitochondrial matrix enzyme that converts glutamate to α-ketoglutarate, a key intermediate in the tricarboxylic acid cycle. Gdh1 expression showed a lower expression during the dark phase for HFD-fed animals (Fig. 5B). Moreover, both genes show a significant Interaction effect between time and diet (Table 1). These data indicate a decrease in conversion of glutamate during the active state of the animals. Microglial activation leads to production of reactive oxygen species (ROS), therefore self-produced antioxidants could have a protective role in the cells. Expression of glutathione peroxidase 1 (Gpx1) – an important antioxidant enzyme, involved in reduction of organic hydroperoxides and hydrogen peroxide by glutathione, showed an inverted pattern of expression during the light phase between both groups (Fig. 5C), suggesting a change in this protective mechanism. No significant daily rhythmicity according to JTK_Cycle analysis was observed for any of the genes under control and obesogenic conditions (see Supplementary Table S2). Together, these data point to an overall decrease of glutamate utilization during the active period of HFD-fed animals.

**Decrease of microglial glucose utilization during the dark phase during HFD.**

It has been shown that glycolysis is crucial for immune cell function (O’Neill, Kishton et al. 2016). Moreover, it has been suggested that upregulation of expression of glycolytic genes leads to M1 polarization in macrophages, known for its proinflammatory function (Wang, Liu et al. 2017). To assess the involvement of glucose metabolism in microglial immune function when rats are fed HFD, we evaluated gene expression of hexokinase 2 (Hk2) - the first glycolytic enzyme converting glucose to glucose-6-phosphate. We observed a decrease of Hk2 expression during the dark phase (ZT18-22) for animals fed HFD, suggesting a decrease in glucose utilization in microglial cells (Fig. 5D). Moreover, there was a gain of rhythm for Hk2 in animals, fed HFD (see Supplementary Table S2). To investigate this further, we evaluated the expression of glucose transporter type 5 (Glut5) – a fructose transporter, which is known to be highly specific for microglial cells (Payne, Maher et al. 1997).
Figure 5. HFD effect on glutamate, glucose and lipid microglial metabolism in rats. Relative gene expression of (top) glutamate substrate utilization genes Gls (A) and Gdh (B), as well as antioxidant enzyme gene Gpx1 (C); (middle) glucose metabolism genes Hk2 (D) and Glut5 (E), fatty acid sensing gene Cd36 (F); (bottom) fatty acid sensing genes Lpl (G) and Ppard (H), as well as fatty acid synthesis gene Fas (I) in HFD-fed rats (red) compared to Chow-fed controls (black) evaluated at six time points, starting at ZT2. Data are presented as means ± SEM. Statistical significance was determined using Two-way ANOVA effect for Interaction, Diet and Time (ZT); Student t-test is used for diet effect within a separate time point (p<0.05*; p<0.01**; p<0.001***). Scale (bottom right) represents light (ZT0-12) and dark (ZT12-24) phase.

a similar trend for Glut5 in HFD-fed animals, with a steady decreased expression towards the end of the dark phase ZT22 (Fig. 5E). Both genes show a significant Interaction effect between time and diet (Table 1). Together these data on glutamate and glucose metabolism, suggest that under obesogenic conditions microglial cells switch their substrate utilization to other sources during their active state.

HFD leads to an increase in lipid utilization and sensing in microglia during the light phase. Fatty acid oxidation can contribute 20% of total brain energy production (Ebert, Haller et al. 2003). A recent study has shown that microglial cells determine hypothalamic inflammation in response to excess saturated fat intake through a direct and specific sensing mechanism
To assess microglial fatty acid (FA) metabolism in DIO, we evaluated genes involved in FA substrate utilization and sensing. Expression of cluster of differentiation 36 (Cd36) – a FA translocase responsible for import of FA inside the cell, showed a flattening of the time-of-day differences in animals fed HFD, compared to controls (Fig. 5F). Evaluation of daily rhythmicity of Cd36 gene expression confirms this observation, with a loss of rhythm under obesogenic conditions (see Supplementary Table S2). This suggests an overall steady import of FA during the day/night cycle under HFD. Previous research from our group has shown that HFD stimulates the expression of microglial lipoprotein lipase (Lpl) - a triglyceride hydrolase receptor involved in receptor-mediated lipoprotein uptake, and that lack of LPL impairs microglial immune reactivity (Gao, Vidal-Itriago et al. 2017). Here, we show that this increase of Lpl expression takes place during the light phase in animals fed HFD (Fig. 5G). These data highlight LPL as a key player in microglial immunometabolism in DIO. Peroxisome proliferator-activated receptors (PPARs) have an important physiological role in lipid sensing and regulation of lipid metabolism during normal healthy conditions, as well in the development of pathologies like obesity and type two diabetes (and and Moller 2002). PPAR delta (Ppard) is highly expressed by microglia and its activity increases oxidative capacity. Our results showed an inverted pattern of Ppard day/night expression in obesogenic animals, with highest expression during ZT2, but lowest at ZT22 (Fig. 5H). To assess the effect of HFD-induced obesity on fatty acid synthesis we evaluated gene expression of fatty acid synthase (Fas) – a key enzyme catalyzing the synthesis of palmitate from malonyl coenzyme A. Fas expression in microglia from HFD-fed animals showed a lower expression at the end of the dark phase and higher expression at the beginning of the light phase, compared to control chow-fed animals (Fig. 5I). These data suggest a shift of FA synthesis to the light phase in HFD-fed animals.

Taken together, these data suggest an overall increase in lipid metabolism during the light, i.e., sleep, phase of animals fed HFD. This increase could be partially explained by the higher levels of non-esterified fatty acids (NEFA) in HFD-fed rodents during the light phase (Fig.2F) (Stavinoha, RaySpellicy et al. 2004, Shostak, Meyer-Kovac et al. 2013). Moreover, we observed a decrease in glutamate and glucose utilization as shown above. This could suggest a microglial metabolic switch to lipid substrate utilization in HFD-induced obesity.

**HFD increases mitochondrial bioenergetics and dynamics gene expression during the light phase.**

To assess whether microglial mitochondria bioenergetics are affected by DIO, we evaluated the gene expression of cytochrome c oxidase subunit 4 (Cox4), encoding a terminal enzyme of the mitochondrial respiratory chain that catalyzes the reduction of oxygen to water, and ATP synthase subunit beta (Atp5b) – encoding a part of the enzyme, catalyzing ATP synthesis. We observed a decrease in Cox4 and Atp5b expression in animals fed HFD at ZT18 (dark...
phase), but an increase during the beginning of the light phase (ZT2), suggesting a shift of energy production to the resting state in obese animals (Fig. 6A&B). These data are in line with our observation on lipid metabolism; therefore, we selected another mitochondrial target, involved in FA metabolism. Pyruvate dehydrogenase kinase 4 (Pdk4) is an enzyme located in the mitochondrial matrix, inhibiting the pyruvate dehydrogenase complex and exerting a regulatory function on substrate utilization by suppressing glycolysis and enhancing FA oxidation. Pdk4 expression showed the same trend for HFD-fed animals, with an increase at ZT2 (beginning of the light phase) (Fig. 6C). This has also been previously observed in heart tissue and soleus muscle of rats fed HFD (Stavinoha, RaySpellicy et al. 2004). Moreover, all three genes show a daily rhythm under control conditions, which was lost in HFD-fed animals, suggesting that hypercaloric diet impairs time-of-day mitochondrial bioenergetics in microglial cells (see Supplementary Table S2).

To test if this trend was also observed in mitochondrial dynamics, as they adjust to mitochondrial demand, we evaluated key genes involved in mitochondrial fusion – mitofusin 2 (Mfn2) and optic atrophy 1 (Opa1); as well as mitochondrial fission – fission 1 (Fis1) and dynamin-related protein 1 (Drp1). Results were supportive of changes in the bioenergetics state, with a significant increase of expression for all four genes (Mfn2, Opa1, Fis1, Drp1) at ZT2 for HFD-fed animals (Fig. 6D-G). Two-way ANOVA test showed a significant Interaction effect for all four genes (Table 1).

Taken together these data suggest an increased energy production in microglia of DIO animals during the light phase, which could be explained by an increased demand to sustain the increase in lipid metabolism. Another recent study indeed showed that mitochondrial fission is elevated as a consequence of high-fat concentrated diets (Putti, Sica et al. 2015). This indicates that mitochondrial dynamics adapt to changes in the bioenergetics state in response to nutritional status.

**The effect of HFD-induced obesity on blood monocyte immunometabolism is less robust than on brain microglial cells**

Following our observations in microglia, we were interested if the same effects could be seen in monocytes – peripheral myeloid cells. Originating from hematopoietic stem cells in the bone marrow, monocytes circulate in the blood and migrate to other tissue where they differentiate into tissue resident macrophages. It is known that under obesogenic conditions, circulating monocytes could infiltrate adipose tissue, leading to macrophage activation and increasing proinflammatory activity (Xu, Barnes et al. 2003, Ghanim, Aljada et al. 2004, Krinninger, Ensenauer et al. 2014).

Our results indicated an overall loss of daily rhythmicity of circadian gene expression, with Clock, Per2 and Dbp showing daily rhythmicity in control animals, which was only maintained
Figure 6. Microglial mitochondria signaling during DIO. Relative gene expression of mitochondria bioenergetics genes Cox4 (A), Atp5b (B) and Pdk4 (C), as well as mitochondria dynamics gene Fis1 (D), Drp1 (E), Mfn2 (F) and Opa1 (G) in HFD-fed rats (red) compared to Chow-fed controls (black) evaluated at six time points, starting at ZT2. Data are presented as means ± SEM. Statistical significance was determined using Two-way ANOVA effect for Interaction, Diet and Time (ZT); Student t-test is used for diet effect within a separate time point (p<0.05*; p<0.01**). Scale (bottom right) represents light (ZT0-12) and dark (ZT12-24) phase.

for Per2 gene expression under obesogenic conditions (see Supplementary Table S2). Bmal1 and Per1 showed a significant increase in expression at the beginning of the light phase (ZT2) in HFD-fed animals compared to control chow (Fig. 7A&C). Gene expression of Reverba and Dbp in monocytes showed a higher expression at ZT6 in HFD-fed animals (Fig. 7E&F). There was no difference in Cry1 and Cry2 gene expression between both conditions (see Supplementary Figure S4A&B). Moreover, One-Way ANOVA analysis showed lack of Time effect for all circadian genes during HFD (see Supplementary Table S3).

We did not find any difference in monocyte immune response between both groups for Tnfa, Ikbkb, Cd68 and Sirt1 gene expression (see Supplementary Figure S4C-F). However, we did observe a daily rhythm in Tnfa and Cd68 in control animals, as well as gain of rhythm for Sirt1 gene expression in HFD-fed animals (see Supplementary Table S2). There was an increase in Il1b expression at ZT2 for the HFD group (Fig. 7G). Il1b showed daily rhythmicity under control conditions, which was maintained under obesogenic conditions with a shift in acrophase of six hours (see Supplementary Table S2). Il1b-induced inflammation has been shown to be indirectly involved in insulin resistance in type 2 diabetes (Ehses, Lacraz et al. 2009, Bing 2015). Thus, these data could indicate a reduction in insulin sensitivity. Moreover, we observed an increased expression of Myd88 at ZT2 for HFD-fed animals (Fig. 7H).
Figure 7. Monocyte immunometabolism in DIO. Relative gene expression of circadian genes Bmal1 (A), Clock (B), Per1 (C), Per2 (D), Dbp (E) and Reverba (F); immune genes Il1b (G) and Myd88 (H), antioxidant enzyme gene Gpx1 (I), glycolysis gene Hk2 (J), fatty acid sensing gene Cd36 (K) and mitochondria dynamic genes Opa1 (L) and Drp1 (M) in HFD-fed rats (red) compared to Chow-fed controls (black) evaluated at six time points, starting at ZT2. Data are presented as means ± SEM. Statistical significance was determined using Two-way ANOVA effect for Interaction, Diet and Time (ZT); Student t-test is used for diet effect within a separate time point (p<0.05*; p<0.01**; p<0.0001****). Scale (bottom right) represents light (ZT0-12) and dark (ZT12-24) phase.

No differences between obese and control animals were found for representative genes of the glutamate pathway Gls and Gdh (see Supplementary Figure S4G-H). However, there was a gain of daily rhythm for Gls gene expression in HFD-fed animals (see Supplementary Table S2). We found an increase in Gpx1 expression at ZT2 for HFD group with an overall stable day/night expression, suggesting a mechanism of constant anti-oxidant production (Fig. 7I). This observation was supported by a loss of daily rhythmicity under obesogenic conditions (see Supplementary Table S2). Expression of the glucose metabolic gene Hk2 was decreased at ZT22 in HFD-fed animals, similar to what was observed in microglia (Fig. 7J). We observed no difference in FA metabolism and sensing genes Fas and Ppard (see Supplementary Figure S4I-J), apart from Cd36 expression (Fig. 7K). Cd36 expression showed a strong daily rhythm under control conditions, which was significant also in HFD-fed animals with an acrophase shift of six hours (see Supplementary Table S2). The expression of the FA translocase in monocytes has also been shown to be associated with insulin resistance, supporting our observation for Il1b expression (Love-Gregory and Abumrad 2011). Lpl evaluation showed low expression (data not shown).
We observed no difference in mitochondrial bioenergetics gene expression between both dietary groups for *Atp5b, Atp5g* and *Cox4* (see Supplementary Figure S4K-M). Mitochondria dynamics gene expression was affected only at ZT2 for *Opa1* and *Drp1* expression (Fig. 7L&M), with no difference in *Mfn2* expression (see Supplementary Figure S4N) and low expression of *Fis1* (data not shown). Interestingly, HFD led to a decrease in mitochondrial bioenergetics gene expression in monocytes at the start of the inactive period, opposite to the increase we observed in microglia under obesogenic conditions. We found no daily rhythm for any of the mitochondria genes, both under control and obesogenic conditions (see Supplementary Table S2). One-Way ANOVA analysis showed lack of *Time* effect for all genes both during control and HFD (see Supplementary Table S3). Two-way ANOVA analysis data is shown the in Supplementary Material (see Table S4). Overall, these data suggest a small effect of the obesogenic diet on monocyte immunometabolism, suggesting that HFD specifically affects microglial immunometabolism.

**Discussion:**

It is well known now that a hypercaloric environment is a potent inducer of microglial activation, which ultimately leads to chronic neuroinflammation (Gao, Ottaway et al. 2014, Valdearcos, Robblee et al. 2014, Baufeld, Osterloh et al. 2016, Gao, Bielohuby et al. 2017). However, the daily rhythm of microglial innate immune function is poorly known, both in obesity and health. The purpose of this study was to evaluate the effect of an obesogenic diet on daily changes in microglial immunometabolism. We evaluated the expression of key genes involved in circadian, immune, metabolic and mitochondrial profile in microglial cells (Fig. 7). Our data showed a disturbance of the microglial interaction between metabolism and immunity during DIO. We report that HFD-induced obesity leads to loss of daily rhythm of circadian genes and impaired microglial immunometabolic functions primarily at the transition period between dark and light phase (ZT22-ZT2).

To evaluate the effect of DIO on daily rhythms in microglial function and activity, we studied the microglial expression of major circadian and immune genes. Under normal conditions, microglia circadian genes were expressed in a rhythmic manner, which is disturbed by HFD, mainly due to a loss of its rhythmicity. Comparable changes have also been observed in different peripheral tissues like liver, brown adipose tissue and skeletal muscle in animals on an obesogenic diet (Wang, Xue et al. 2013, Branecky, Niswender et al. 2015, de Goede, Sen et al. 2018). However, to our knowledge, we are the first to report an effect of HFD on expression rhythms of microglial clock genes. The presently reported difference in time-of-day expression of microglial cytokine genes, is in line with our previous results (Yi, Walter et al. 2017). Fonken et al. have shown previously that *Il1b* and *Tnfa* gene expression have a peak during the middle of the day, contrary to our observations (Fonken, Frank et al. 2015). Possible explanation to this contradiction is the heterogeneous transcriptional identities of...
microglia, specific for each brain region, in this case hippocampal versus cortical microglia (Grabert, Michoel et al. 2016).

Microglial cells are known to exhibit bioenergetics shifts in energy substrate, for example during aging (Flowers, Bell-Temin et al. 2017). Such a shift in substrate utilization is known to have an effect on the activation status of immune cells (Kelly and O’Neill 2015, O’Neill, Kishton et al. 2016). We studied microglial substrate utilization, focusing on glutamate, glucose and FA metabolism and observed a difference between control and HFD-fed animals, particularly during the transition period from the dark to light phase. Key players in the glutamate pathway have been shown to be involved in macrophage immune function, e.g. glutamine availability was shown to modulate macrophage phagocytic capacity, while α-ketoglutarate, generated through glutaminolysis, is crucial in eliciting an anti-inflammatory phenotype in macrophages (de Oliveira, da Silva Lima et al. 2016, Liu, Wang et al. 2017). We report a decrease in microglial glutamate utilization in the active period of HFD-fed animals as seen in glutamine conversion to glutamate and glutamate conversion to α-ketoglutarate. Additionally, a similar change was observed for glucose metabolism with decreased glucose utilization in the active period of HFD-fed animals. However, we observed an increase in FA sensing and synthesis at the beginning of the light period under obesogenic conditions, suggesting a shift to FA utilization during the sleep phase of the animal. It has been shown that FA treatment of BV2 cells (a microglial cell line) is a potent inducer of cytokine production via TLR4 signaling, thus leading to low-grade inflammation even in the absence of immune challenge (Button, Mitchell et al. 2014). This FA metabolism increase could be a possible explanation for our previously observed constant day/night activation of hypothalamic microglia under HFD (Thaler, Yi et al. 2012). Additionally, we know that immune cell activation requires higher energy production. We here show that microglial mitochondrial function in DIO is increased during the inactive period, suggesting an increase in ATP production, which could be explained by the increased FA metabolism demand. These data support the view that mitochondrial function adapts to nutritional status (Putti, Sica et al. 2015).

To investigate whether the observed effect of HFD on immunometabolism is restricted to microglial cells, we also studied monocyte immunometabolism in obesity. We report small or no effect of the hypercaloric diet on monocyte immunometabolic function, which suggests a microglia-specific functional disturbance in HFD-induced obesity. Taken together, our data suggest that microglial innate immunity is highly dependent on metabolic changes, as well as the time of day. Microglial cells are highly active cells, with a high energy demand, which is achieved by a strictly regulated cellular metabolism. A robust switch of substrate utilization is a suitable mechanism, in response to the high demands of immune defense.

The data currently presented suggest a deleterious effect of an obesogenic diet on microglial function by inducing chronic activation. It has been shown that chronic microglial activation has a negative impact on neuronal function and could play a role in obesity-associated
cognitive decline (Valdearcos, Robblee et al. 2014, Cope, LaMarca et al. 2018). Our data point out to the importance of microglial integrity and the negative impact of chronic exposure to a hypercaloric environment on cortical microglial function, which could ultimately lead to cognitive impairment. Previously we observed that obesity induces microglial activation in close proximity to the anorexigenic proopiomelanocortin (POMC) neurons located in the arcuate nucleus of the hypothalamus (Yi, Walter et al. 2017). Moreover, chronic HFD feeding leads to POMC neuronal loss, which would lead to further progression of obesity (Thaler, Yi et al. 2012). It is possible that the current observation on cortical microglia could be translated to the hypothalamus, which would give insight in the mechanisms behind this neuronal loss. Finally, three issues need to be addressed: firstly, we observed a clear effect of HFD on microglial immunometabolism, leading to an increase in expression of many of the presented genes around the end of the dark period, i.e., ZT22/ZT2. In order to check whether or not a higher food intake at the end of the dark period in the HFD-fed group could be responsible for these changes, we re-analyzed the food intake data from metabolic cage experiments from a separate cohort of rats fed a similar HFD (Stenvers, van Dorp et al. 2016). With respect to consumed grams, no difference in timing of food intake was found between control and obesogenic diet (see Supplementary Figure S6). However, with respect to consumed calories, the obesogenic diet group showed a larger increase of kcal intake at the beginning and the end of the dark period, although only significant for the beginning of the dark period, suggesting that higher energy consumption (but not higher food intake) may be partially responsible for the differences in gene expression between the HFD and control group at the end of the dark period (see Supplementary Figure S6). Secondly, we cannot distinguish between the effect of obesity and the hypercaloric diet itself. However, a hypercaloric diet can induce microglial activation in the hypothalamus after one day, prior to any changes in body weight, pointing to an effect of diet rather than obesity itself (Waise, Toshinai et al. 2015). Thirdly, the data presented only show the transcriptional state of selected target genes, representative of the different functions investigated. Future studies should be aimed at a further understanding of activity changes in each of the represented pathways.

Conclusions:
An obesogenic diet affects microglial immunometabolism in a time-of-day specific manner. The aim of this study was to increase the knowledge of microglial cell function in obesity in general and its daily rhythms in specific. To our knowledge, we are the first to point out (loss of) time-of-day differences for microglial cells during HFD. Our data are supportive of the ongoing research, focused on the interaction between immune cells and metabolism. Further studies should focus on addressing the time-of-day differences in microglial function, as more detailed knowledge of microglial immunometabolism could lead to a better understanding of the neuroinflammatory process taking place in the CNS under chronic hypercaloric environment.
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References


Mariani MM, Kielian T. Microglia in infectious diseases of the central nervous system. Journal of neuroimmune pharmacology: the official journal of the Society on


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Table 1. Two-way ANOVA assessment of effect of Time, Diet and Interaction in microglia. Diet, Time and Interaction effects were evaluated in microglia for circadian, inflammatory, metabolic and mitochondrial genes. Statistical significance was determined using Two-way ANOVA effect for Interaction, Diet and Time (ZT). Data are presented as means ± SEM. Genes are considered rhythmic when p<0.05 (Bold).
Figure S1. Monocyte immunometabolism in obesity – additional data. Relative gene expression of circadian genes *Cry1* (A) and *Cry2* (B); innate immunity genes *Tnfa* (C) and *Ikbkb* (D), phagocytic indicator gene *Cd68* (E), *Sirt1* gene (F); glutamate substrate utilization genes *Gls* (G) and *Gdh* (H); fatty acid synthesis gene *Fas* (I) and fatty acid sensing gene *Ppard* (J); mitochondria bioenergetics genes *Atp5b* (K), *Atp5g* (L) and *Cox4* (M) and mitochondria dynamics gene *Mfn2* (N) in HFD-fed rats (red) compared to Chow-fed controls (black) evaluated at six time points, starting at ZT2. Data are presented as means ± SEM. Statistical significance was determined using Two-way ANOVA effect for *Interaction, Diet* and *Time* (ZT); Student t-test is used for diet effect within a separate time point (p<0.05*). Scale (bottom right) represents light (ZT0-12) and dark (ZT12-24) phase.
Figure S2. Average 24h food intake

Figure S2. Average time-of-day food intake per hour. Graphs represented show a re-analysis of food intake data from metabolic cages for rats fed a HFD (4.7kcal/g) or control diet (3.1 kcal/g) (Stenvers, van Dorp et al. 2016). Data show average food intake per hour starting at ZT0. Data are presented as food intake in gram (p_int = 0.7440, p_time <0.0001, p_diet = 0.1001) (left) and kcal (p_int = 0.0033, p_time <0.0001, p_diet = 0.0003) (right), as 24h mean of 48h measurement for control (black) and HFD (orange) groups (n=8). Data are presented as means ± SEM. Statistical significance was determined using Two-way ANOVA effect for Interaction, Time (ZT) and Diet; Sidak’s multiple comparison test was used to compare the effect of diet for each time point (p<0.05*; p<0.0001****).
Table S1. Primer sequences of target genes

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<td>TGCCAGTTTCTCTGTCGA</td>
</tr>
<tr>
<td>Fis1</td>
<td>GGTGATGATGTGTAAGGGGTA</td>
<td>CTGFAACAGTCTGCCCACAT</td>
</tr>
<tr>
<td>Drp1</td>
<td>ACAAACAGGAGAAGAAAATGAGATTG</td>
<td>GTGGGGGCAGAAACCTTT</td>
</tr>
<tr>
<td>Mfn2</td>
<td>CTCAAGGAGCCACGCGTTATTGT</td>
<td>TGTCAGGGACAGCATGCTCAT</td>
</tr>
<tr>
<td>Opa1</td>
<td>AAGGCAATCCACCACACAGGA</td>
<td>CCTGCGAATGATTCGTCGTC</td>
</tr>
<tr>
<td><strong>Housekeeping</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hpirt (origin: Mus musculus)</td>
<td>GCAGTACAGCCCAAAATGG</td>
<td>AACAAGTCTGGCTGTATCCCA</td>
</tr>
<tr>
<td>bactin</td>
<td>ACAACTCTTTGTCAGTCCTC</td>
<td>CTGACCATAACCCACATCAC</td>
</tr>
</tbody>
</table>

*Two different primers for Gpx1 were used for monocyte and microglial cells as the primers showed tissue specificity and a single primer, suitable for both cell types was not found during optimization of the technique.
### Table S2. Daily rhythmicity analysis

<table>
<thead>
<tr>
<th>Genes</th>
<th>JTK_Cycle analysis for microglia</th>
<th>JTK_Cycle analysis for monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p-value</td>
<td>acrophase</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td><em>Bmal1</em></td>
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<td>2</td>
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<tr>
<td><em>Clock</em></td>
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<td>NR</td>
</tr>
<tr>
<td><em>Cry1</em></td>
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<td>16</td>
</tr>
<tr>
<td><em>Cry2</em></td>
<td>1</td>
<td>NR</td>
</tr>
<tr>
<td><em>Per1</em></td>
<td>0.001</td>
<td>14</td>
</tr>
<tr>
<td><em>Per2</em></td>
<td>0.003</td>
<td>14</td>
</tr>
<tr>
<td><em>Reverba</em></td>
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<tr>
<td><em>Dbp</em></td>
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<td>12</td>
</tr>
<tr>
<td><strong>Inflammatory</strong></td>
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<td></td>
</tr>
<tr>
<td><em>Tnfa</em></td>
<td>1</td>
<td>NR</td>
</tr>
<tr>
<td><em>Il1b</em></td>
<td>0.07</td>
<td>NR</td>
</tr>
<tr>
<td><em>Myd88</em></td>
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<td>4</td>
</tr>
<tr>
<td><em>Ikbkb</em></td>
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<td>NR</td>
</tr>
<tr>
<td><em>Cd68</em></td>
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<td>NR</td>
</tr>
<tr>
<td><em>Sirt1</em></td>
<td>1</td>
<td>NR</td>
</tr>
<tr>
<td><strong>Metabolic</strong></td>
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<td></td>
</tr>
<tr>
<td><em>Gls</em></td>
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<td>NR</td>
</tr>
<tr>
<td><em>Gdh</em></td>
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<td>NR</td>
</tr>
<tr>
<td><em>Gpx1</em></td>
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<td>NR</td>
</tr>
<tr>
<td><em>Hk2</em></td>
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<td>NR</td>
</tr>
<tr>
<td><em>Glut5</em></td>
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<td>NR</td>
</tr>
<tr>
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<tr>
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<td>NR</td>
</tr>
<tr>
<td><em>Ppard</em></td>
<td>1</td>
<td>NR</td>
</tr>
<tr>
<td><em>Fas</em></td>
<td>1</td>
<td>NR</td>
</tr>
<tr>
<td><strong>Mitochondrial</strong></td>
<td></td>
<td></td>
</tr>
<tr>
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<tr>
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<tr>
<td><em>Atp5g</em></td>
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<td>N/A</td>
</tr>
<tr>
<td><em>Pdk4</em></td>
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<td>14</td>
</tr>
<tr>
<td><em>Fis1</em></td>
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<td>NR</td>
</tr>
<tr>
<td><em>Drp1</em></td>
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<td>NR</td>
</tr>
<tr>
<td><em>Mfn2</em></td>
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<td>NR</td>
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<tr>
<td><em>Opa1</em></td>
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<td>NR</td>
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Table S2. JTK_Cycle analysis of daily rhythmicity. Diet and Time effect on daily rhythms in microglia and monocytes for circadian, inflammatory, metabolic and mitochondrial genes. Data was analyzed with JTK_Cycle software and p-values were obtained by fitting the data on a curve with fixed 24h period. The acrophase is given for rhythmic genes (in ZT). Genes are considered rhythmic when p<0.05 (Bold). N/A = not applicable (gene has low or no expression); NR = not rhythmic.
<table>
<thead>
<tr>
<th>Genes</th>
<th>One-way ANOVA analysis of effect of <em>Time</em> for microglia</th>
<th>One-way ANOVA analysis of effect of <em>Time</em> for monocytes</th>
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<tbody>
<tr>
<td></td>
<td>Chow</td>
<td>HFD</td>
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<tr>
<td><strong>Circadian</strong></td>
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</tr>
<tr>
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<td>0.0004</td>
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<tr>
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<td>0.0148</td>
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<tr>
<td><em>Cry1</em></td>
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<td>0.0018</td>
</tr>
<tr>
<td><em>Cry2</em></td>
<td>0.0012</td>
<td>0.0202</td>
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<tr>
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<tr>
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<td>0.0004</td>
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<td></td>
</tr>
<tr>
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<tr>
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<td>N/A</td>
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<td>0.1677</td>
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Table S3. One-way ANOVA assessment of effect of *Time*. Time effect evaluation in separate feeding groups. Statistical significance was determined using One-way ANOVA effect *Time* (ZT). Genes are considered rhythmic when *p*<0.05 (**Bold**). N/A = not applicable (gene has low or no expression).
Table S4. Two-way ANOVA analysis of monocytes

<table>
<thead>
<tr>
<th>Genes</th>
<th>Two-way ANOVA analysis</th>
<th>Genes</th>
<th>Two-way ANOVA analysis</th>
</tr>
</thead>
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<td></td>
<td>p-value</td>
<td></td>
<td>p-value</td>
</tr>
<tr>
<td></td>
<td>Interaction</td>
<td>Time</td>
<td>Diet</td>
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<td></td>
<td></td>
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<tr>
<td><em>Bmal1</em></td>
<td>0.0401</td>
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<td>0.1016</td>
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<td>&lt;0.0001</td>
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</tr>
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<td>0.3303</td>
<td>0.2993</td>
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Table S4. Two-way ANOVA assessment of effect of Time, Diet and Interaction in monocytes. Statistical significance was determined using Two-way ANOVA effect for Interaction, Diet and Time (ZT). Data are presented as means ± SEM. Genes are considered rhythmic when p<0.05 (Bold).
The epidemic of the metabolic syndrome, with obesity and type 2 diabetes mellitus (T2DM) at its core, represents one of the most severe health threats of modern society, a threat that will cause an unprecedented economic burden in the future if not stopped. Current knowledge implicates impaired brain control over food intake and energy metabolism as a key pathogenic process leading to obesity and T2DM. The neural and neuroendocrine outputs from the hypothalamus that are in control of metabolism are mediated by diverse neural populations, such as the orexigenic AgRP/NPY and the appetite-curbing POMC neurons located in the mediobasal hypothalamic IFN, and the pre-autonomic neurons located in paraventricular nucleus (PVN). While most research has concentrated on impaired neural networks and/or neuroendocrine mechanisms, to search for targets of treating obesity and T2DM, recent studies also found that not only the key metabolic regulatory neurons, but also the previously largely neglected “supporting cells”, in particularly the microglia surrounding the neurons, are important players in the hypothalamic metabolic regulatory machinery (Thaler, Yi et al. 2012, Gao, Ottaway et al. 2014, Kim, Suyama et al. 2014, Garcia-Caceres, Quarta et al. 2016). Microglia are the innate immune cells responsible for maintaining a homeostatic and “clean” microenvironment, via scavenging debris produced by other cells and secreting cytokines for a proper immune response (Kreutzberg 1996). Microglia in the ARC respond to a high-carbohydrate high-fat (HCHF) diet (mimicking a human western-style hypercaloric diet) by significantly increasing their reactivity (Thaler, Yi et al. 2012, Gao, Ottaway et al. 2014). This reactivity is followed by astrocytosis and angiogenesis in the same area (Thaler, Yi et al. 2012, Yi, Gericke et al. 2012). These data indicate that on a HCHF diet, all cell types within the hypothalamic microenvironment start to lose their integrity as a joint unit. Eventually this results in a vicious cycle of cellular destruction ending with POMC neuronal dysfunction in the control of food intake and energy expenditure.

Knowledge on the intracellular mechanism of the reactive microglia and the interactions between the reactive microglia, neurons and other cells in the microenvironment is still sparse, and the majority of the studies on these questions have been performed in animal models. However, there are some major obstacles that hamper translational studies on brain dysfunction in T2DM patients at the molecular level. First of all, among the different types of animal models applied to mimic human T2DM, the most profound diabetic pathogenesis is obtained in genetic models, such as the Zucker diabetic fatty rats and the db/db mice that both have a mutation in the leptin receptor (Lee, Proenca et al. 1996, Takaya, Ogawa et al. 1996). However, the major causes of human T2DM are not associated with leptin receptor mutations, but with environmental factors in our modern obesogenic society, such as the
excessive consumption of hypercaloric food and a sedated life style. Secondly, although the hypercaloric diets that are commonly used to generate obese animal models do impair glucose metabolism and insulin sensitivity, the transition from this pre-diabetes stage to T2DM that takes place in most of the T2DM patients when disease progresses does not occur in these diet-induced pre-diabetic animals, unless using additional approaches to induce significant β cells loss, for instance by streptozotocin (Skovso 2014). Therefore, thus far there is no perfect animal model that fully mimics human T2DM pathogenesis by environmental factors. Thirdly, although non-invasive brain imaging techniques have provided data on overall changes in brain metabolism in patients with T2DM, it is poorly understood what these changes mean for specific brain regions and individual cells. In this PhD study, we had the opportunity to work with the unique collection of T2DM postmortem brains in the Netherlands Brain Bank, with fully informative medical records. This gave us the opportunity to retrogradely analyse the medical parameters of T2DM patients and matched control subjects, and to systemically study pathological changes in their brains at the protein expression level.

In the different studies presented in this thesis, we made several novel findings. Some of these findings raised new research questions that will need further research efforts in the future. In the study on the neurons and glial cells in the IFN in Chapter 2, we observed a loss of POMC neurons, which has also been described in previous studies (Alkemade, Yi et al. 2012, Li, Tang et al. 2012). The loss of POMC neurons is believed to be one of the major causes of obesity and associated glucose intolerance and insulin resistance in T2DM, due to loss of control on food intake and energy expenditure, which eventually will drive the system into a diabetic stage. Based on previous studies in experimental animals the hypothesis was raised stating that reactive microglia-derived cytokine TNFalpha exerts a detrimental effect on POMC neurons (Yi, Walter et al. 2017). However, in the IFN of T2DM patients, the number of microglial cells was not significantly different from that in the non-diabetic control subjects, thus an alternative hypothesis is needed to explain the POMC neuronal loss. It is known that in the brain, astrocytes are the major nutrient suppliers for their neighboring neurons (Yi, Habegger et al. 2011). One of our hypotheses is that under high fat diet feeding conditions, dysfunctional lipid metabolism takes place during astrogliosis. This high fat diet induced dysfunctional lipid metabolism hampers the regular lipid production in astrocytes and thus causes an abnormal lipid supply to the neighboring POMC neurons. This hypothesis is supported by the evidence that the apoE content was reduced in reactive astrocytes of high fat diet-induced obese/pre-diabetic rats, and that supply of ApoE in the brain can activate POMC gene expression and inhibit food intake (Shen, Tso et al, 2008). Furthermore, a mouse study in which lipoprotein lipase (LPL), the key enzyme hydrolysing triglycerides into fatty acids, was knocked-out in astrocytes, showed accelerated weight gain on a high fat diet and glucose intolerance on both chow and high fat diets (Gao, Layritz et al. 2017). These data suggest that astroglial lipid metabolism might be a key player in maintaining hypothalamic neuronal control of glucose
metabolism. Once astroglial lipid metabolism has become abnormal, the POMC neurons are the most vulnerable population to be affected. How this abnormal lipid supply from astrocytes disturbs the intracellular mechanism of POMC neurons remains as a research question.

An intriguing finding in Chapter 2 with respect to anti-diabetic treatment is the role played by Metformin in the brain. We observed that in T2DM subjects, Metformin treatment was associated with lower NPY-ir, iba1-ir and TMEM119-ir in the NPY regions. Discovered in the 1920’s, Metformin is currently the most widely prescribed anti-diabetic drug in the world. However, the exact pharmacology of Metformin is still to be discovered. A lot of evidence points to a neuroprotective role of Metformin in different disease models (Markowicz-Piasecka, Sikora et al. 2017, Rotermund, Machetanz et al. 2018, Muri, Le et al. 2019). However, in our study, Metformin treatment seems to be associated with an inhibitory or detrimental effect on NPY neurons and microglial cells. Thus, we should keep in mind that in the brain, there might be an unknown and unwanted side effects of the long-term Metformin use in T2DM. It has been reported that in cancer cells, Metformin prevents cell growth by reducing glutamate, ammonia accumulation and autophagy (Saladini, Aventaggiato et al. 2019). In microglial cells, glutamate is an important substrate for glutathione synthesis (Persson, Sandberg et al. 2006), glutathione is one of the body’s most important and potent antioxidants (Battin and Brumaghim 2009). Whether long-term metformin treatment is a cause of excessive oxidative stress and microglial dysfunction is one of the next questions we want to answer.

In the study on the impact of T2DM on the central brain or biological clock in the SCN in Chapter 3, we found that besides the AVP and VIP neurons, also the number of GFAP-ir astroglial cells in the SCN was reduced in T2DM subjects, which suggests that astroglial cells may play an important role in maintaining SCN function. Indeed, two recent studies on astroglial cells in the mouse SCN provided evidence coherent with such an assumption. The study of Tso et al. demonstrated that lack of the core clock gene Bmal1 specifically in astroglial cells within the SCN disturbed the circadian period of locomotor behavior and clock gene expression in the SCN (Tso, Simon et al. 2017). Another study showed that in the absence of other cellular clocks, the cell-autonomous astroglial intracellular transcription-translation negative feedback loops alone could drive molecular oscillations in the SCN and circadian behavior in mice (Brancaccio, Edwards et al. 2019). Interestingly, cultured rat astroglial cells express receptors for AVP (Hosli, Hosli et al. 1991). Therefore, the reduced number of GFAP-ir astroglial cells in T2DM suggests that an intact neuron-glial interaction between the AVP-ir neurons and astroglial cells is necessary for maintaining the molecular oscillations in the human SCN. Thus, studies to specifically address whether human astroglial cells in the SCN express AVP receptor are needed to answer this question.

Another interesting question raised by our studies is how T2DM pathogenesis affects the neurons and glial cells in the SCN. It is known from animal studies that disturbing the temporal distribution of food intake, for instance by ultradian or time-restricted feeding, does not
change the phase of cyclic gene expression in the SCN, but does change AVP expression in the SCN as well as circadian rhythms in peripheral tissues (de Goede, Sen et al. 2018). Indeed, recently we showed an 80% decrease in the number of rhythmic genes in the subcutaneous adipose tissue of patients with T2DM as compared to control subjects (Stenvers, Jongejan et al. 2019). On the other hand, also a mechanism associated with insulin resistance in AVP-ir neurons and astroglial cells might underlie the mechanism that leads to the loss of these cells. Unfortunately, due to plasma sample limitations, we have no data on plasma insulin levels in control subjects or T2DM patients. In T2DM subjects that received insulin treatment, GFAP-ir astroglial cells showed a trend towards a daily rhythmicity. Previously we found that deletion of insulin receptors in astroglial cells results in astroglial dysfunction (Garcia-Caceres, Quarta et al. 2016). Thus, a direct effect of insulin on astroglial cells in T2DM subjects might underlie the gained rhythmicity in these glial cells. Questions that remain are whether AVP-ir neurons also express insulin receptors and whether AVP-ir neurons and astroglial cells in SCN develop insulin resistance in T2DM.

We also observed in our study a seasonal rhythmicity in iba1-ir microglial cells in the SCN (Chapter 3 Supplemental Figure 4). It is known that the innate immune function is enhanced during the winter time to cope with many diseases (Nelson 2004). Our data indicated for the first time that also brain microglial cells might play a critical role in seasonal immunity. The loss of seasonal rhythmicity in the T2DM subjects indicates that brain innate immune function might be disturbed by T2DM pathogenesis. How this disturbance links to SCN dysfunction needs further study. Previously seasonal rhythms in SCN volume, as well as AVP and VIP cell number have been described (Hofman & Swaab, 1992, 1993, 1995; Hofman, 2004). In the present study these rhythms did not reach statistical significance in the control group, probably due to the low number of subjects. Therefore, it is not clear whether T2DM pathogenesis also affects the seasonal rhythms in SCN AVP and VIP content.

Previous studies in animal models also suggested that a hypercaloric diet reduced neural stem cell numbers and their ability to make new neurons in the hypothalamus (Davalos, Ryu et al. 2012, Li, Tang et al. 2012). Thus, we should not exclude the possibility that in both SCN and IFN, where we have observed loss of neurons, there is a potential loss of neurogenesis due to the detrimental effects of T2DM pathogenesis on the neural stem cells. To profile the stem cells in the different hypothalamic regions is one of our future research tasks.

So far, we have only focused on the neurons in the IFN and SCN in the hypothalamus. It is largely unknown what are the changes in the PVN of T2DM patients. The role of PVN neurons, such as those producing arginine vasopressin, oxytocin, corticotropin-releasing hormone, or thyrotropin-releasing hormone, as well as stem cells, in T2DM pathogenesis should not be neglected in future studies.

In Chapter 4, we performed a study on the association between T2DM and AD. Besides an increased risk for AD, T2DM patients also have an increased risk of developing Parkinson’s
disease (PD). Even though these latter results are still disputed - potentially due to varying inclusion/exclusion criteria, clinical assessment, incomplete follow-up data and unidentified confounding factors - the majority of the prospective cohort studies and meta-analyses suggest that diabetes is indeed a risk factor for PD. Supporting this notion is the fact that several agents used in the treatment of T2DM have been shown to be neuroprotective in animal models of PD and are now being evaluated in randomized, controlled trials in patients with PD (i.e., Pioglitazone, Exenatide, and Metformin (Wahlqvist, Lee et al. 2012, Aviles-Olmos, Dickson et al. 2013, Aviles-Olmos, Limousin et al. 2013)). At first glance this “molecular” commonality might be surprising since these diseases present very different in the clinic. PD is characterized by a loss of particularly dopaminergic neurons in the substantia nigra and resulting in specific motor deficits, whereas T2DM patients exhibit insulin resistance combined with a loss of pancreatic beta-cell function and mass. Thus, even though clinically quite disperse, both diseases are characterized by a degenerative phenotype. It will be interesting to investigate the dopaminergic neurons in the substantia nigra of the T2DM patients and whether in these neurons evidence can be found for a mechanistic link between T2DM and PD.

In order to survive, neurons in the central nervous system require a healthy microenvironment, the maintenance of which critically depends on the innate immune cells, the microglia. Microglia can initiate immune protection under physiological or pathological conditions to efficiently clear away metabolic products and cellular debris (Hughes 2012). When microglial activity does not match the demands of the immune defense, a homeostatic and “clean” microenvironment cannot be maintained properly (Kreutzberg 1996) and different aspects of neuronal function may be impaired. Under physiological conditions, the debris in the microenvironment is largely derived from active neurons, especially at the synaptic level (so-called synaptic debris) (Tremblay 2011). Plastic synaptic activity is an essential part of the neurophysiology and plays a key role in the control of energy homeostasis by neurons in the hypothalamus (Yang, Atasoy et al. 2011). Compelling evidence shows that the biological clock is deeply involved in the control of neuronal activity, synaptic plasticity and energy homeostasis. For example, a time-of-day dependent neuronal activity exists in the orexigenic hypothalamic orexin and AgRP neurons, both displaying a robust diurnal rhythmic gene expression pattern with peak activity in the middle of the dark phase, i.e., when rodents are active (Lu, Shieh et al. 2002). Therefore, also in the microenvironment, the need of scavenging and removing neuronal debris will be time-dependent, which thus requires that microglial cleaning activity is temporally synchronized to neuronal activity. Consequently, a desynchronization between the microglia and the neuronal activity will lead to an unhealthy local microenvironment for neural survival. Indeed, previously we showed that in standard chow diet fed lean mice, microglial activity in the arcuate nucleus fluctuates at the morphological level during the day and night, with peak activity and cytokine production in the middle of the dark phase when AgRP neurons are active. However, in high fat diet induced obese mice, this time-of-the-day...
associated rhythm in microglial activity had disappeared, indicating that in a hypercaloric environment, rhythmic microglial activity is impaired (Yi, Walter et al. 2017). Our data in Chapter 6 show how microglial function fluctuates at the gene expression level during the day/night cycle. Furthermore, other studies have shown that the immune response of microglia to lipopolysaccharide is time-of-the-day dependent (Fonken, Frank et al. 2014). All these data suggest that the intrinsic clock machinery in microglia might be a key player in the control of microglial physiology. Based on our findings in Chapter 6, it is logical to hypothesize that the intrinsic molecular clock that controls rhythmic microglial activity is essential for timely clearing-up the debris produced by active neurons and synapses and that this is a common machinery in neural circuits that controls energy homeostasis in the hypothalamus. Therefore, more studies are needed to investigate whether interruption of the biological clock in microglia impairs their phagocytic capacity and immune response to pathological challenges, and whether interruption of the biological clock in microglia impairs synaptic activity in metabolic control circuits of the hypothalamus.

During our exploration of the human T2DM postmortem brain tissue, it became clear that we had to cope with some (obvious) limitations. One of the issues being the large variation in the size of the human brain, which usually is not an issue in animal studies with mostly rather homogenous groups. Along with this variation in brain size also the size of the hypothalamus and individual brain nuclei along the rostral to caudal axis showed considerable variation. This means that a strategy that uses evenly distributed sections is actually biased, because either the total cell number or the average cell number per section will not fairly represent the actual cell distribution along the entire brain region. This means there is no perfect way to compare morphological parameters without bias in topography. We have handled this issue by using neuropeptide stainings, such as NPY or AVP, and the brain sections having the highest level of immunoreactivity as a reference level for the other parameters that have a less clear topographical indication, such as microglia and astrocytes.

Another issue appeared while we were performing GFAP antibody staining. It became clear that the postmortem immersion fixation procedure affected the GFAP-ir astrocytes quite a lot. Due to the immersion fixation, as opposed to the perfusion fixed animal tissue, the outer layers of the brain tissue blocks are fixated first, whereas the inner parts of the tissue block are fixated much later. This difference clearly affected the quality of the astrocyte- / GFAP-epitope in the tissue. Without antigen retrieval, we saw a nice staining at the edges of the tissue, but no staining in the inner part of the tissue. For other antigens this problem usually can be solved by antigen retrieval (heating of the tissue makes the epitopes more accessible for antibodies), but in case of GFAP the antigen retrieval induced a very high background, likely due to unspecific antibody-antigen binding. With this limitation, we could only profile GFAP staining in certain brain regions such as the area around SCN. In addition, we could only perform manual countings of astrocyte cell number, but not quantify their morphology.
because of the high background. In future studies, we will need to establish an alternative approach that allows us to better profile astrocytes in the postmortem human brain tissue. When studying T2DM, it is important to know the duration of the disease. Again, this is not an issue in animal studies when the modeling time for diabetes is controlled experimentally. However, with T2DM in humans it is very difficult to know the actual duration of diabetes, as most of the patients are only diagnosed as such when they already have obvious associated with diabetes, which means that diabetes duration probably is much longer than the time when the diagnosis was made. In the studies presented here we obtained a relative estimation of duration from information on the anti-diabetic treatment. This limitation may thus cause discrepancies between our studies and studies performed in other countries, due to differences in clinical diagnostic criteria, drug availability and medical care systems. Nevertheless, we will continue our research efforts using the unique collection of the Netherlands Brain Bank to explore further the brain mechanism of T2DM and its associated complications in the human brain, bearing these issues in mind.

References
clock.” Brain Behav Immun.
Metformin in Neurodegenerative Diseases.” Front Endocrinol (Lausanne) 9: 400.
Saladini, S., M. Aventaggiato, F. Barreca, E. Morgante, L. Sansone, M. A. Russo and M.
Tafani (2019). “Metformin Impairs Glutamine Metabolism and Autophagy in Tumour
Cells.” Cells 8(1).
“Brain apolipoprotein E: an important regulator of food intake in rats”. Diabetes 57(8):
2092-2098.
Stenvers, D. J., A. Jongejan, S. Atiqi, J. P. Vreijling, E. J. Limonard, E. Endert, F. Baas, P.
the white adipose tissue transcriptome are disturbed in obese individuals with type 2
diabetes compared with lean control individuals.” Diabetologia.
Takaya, K., Y. Ogawa, N. Isse, T. Okazaki, N. Satoh, H. Masuzaki, K. Mori, N. Tamura,
complementary DNAs--identification of a missense mutation in Zucker fatty (fa/fa)
Thaler, J. P., C. X. Yi, E. A. Schur, S. J. Guyenet, B. H. Hwang, M. O. Dietrich, X. Zhao,
D. A. Sarruf, V. Izgur, K. R. Maravilla, H. T. Nguyen, J. D. Fischer, M. E. Matsen, B.
Invest 122(1): 153-162.
“Astrocytes Regulate Daily Rhythms in the Suprachiasmatic Nucleus and Behavior.”
“Metformin-inclusive sulfonylurea therapy reduces the risk of Parkinson’s disease
occurring with Type 2 diabetes in a Taiwanese population cohort.” Parkinsonism Relat
Disord 18(6): 753-758.
memory circuit via a synaptic AMPK-dependent positive feedback loop.” Cell 146(6):
992-1003.
Yi, C. X., M. Gericke, M. Kruger, A. Alkemade, D. G. Kabra, S. Hanske, J. Filosa, P.
Pfluger, N. Bingham, S. C. Woods, J. Herman, A. Kalsbeek, M. Baumann, R. Lang, J. E.
astrocytes in the central control of metabolism.” Neuroendocrinology 93(3): 143-149.
Yi, C. X., M. Walter, Y. Gao, S. Pitra, B. Legutko, S. Kalin, C. Layritz, C. Garcia-Caceres,
M. Bielohuby, M. Bidlingmaier, S. C. Woods, A. Ghanem, K. K. Conzelmann, J. E.
POMC neurons in obesity.” Nat Commun 8: 15143
Summary

The main goal of this thesis was to investigate neurons and glial cells in the forebrain of metabolic disordered humans, with obesity and (pre)diabetes at its core. In Chapter-1, an overview is provided of the peptidergic neurons and glia cells in the infundibular nuclei (IFN) in the mediobasal hypothalamus in overeating, obesity and diabetes, as this brain region is the most important hypothalamic region that controls energy homeostasis, food intake and body weight. We focused on how peripheral hormonal and nutrient signals, such as leptin, insulin, glucose and fatty acids, act on the peptidergic neurons and glial cells of the IFN. In addition, we highlighted the main intra-hypothalamic projection sites of the IFN.

Following Chapter-1, we investigated how neurons and glial cells in different regions of the human brain are affected by the disease process of T2DM. We focussed on brain regions involved in the control of energy homeostasis, (Chapter-2) circadian rhythms (Chapter-3) and learning and memory (Chapter-4). In Chapter-2, the changes in proopiomelanocortin (POMC) and neuropeptide-Y (NPY) immunoreactive (-ir) neurons, and the ionized calcium-binding adapter molecule 1 (Iba1)-ir and transmembrane protein 119 (TMEM119)-ir microglia in the IFN of T2DM subjects with different anti-diabetic treatments and matched non-diabetic control subjects was characterized. We found a significant decrease in the number of POMC-ir neurons, but no changes in NPY-ir neurons or microglia when comparing T2DM subjects and matched control subjects. Interestingly, T2DM subjects treated with the anti-diabetic drug Metformin had less NPY-ir neurons and less microglia compared to T2DM subjects without metformin treatment. We also found that the number of microglia correlated with the number of NPY-ir neurons, but only in T2DM subjects. These results indicate that in the IFN different changes in hypothalamic neurons and microglial cells accompany T2DM. These highly selective changes in hypothalamic neurons and microglial cells were dependent on the T2DM treatment modality.

The IFN in the posterior hypothalamus has mutual interactions with the suprachiasmatic nucleus (SCN), i.e. the central biological clock, in the anterior hypothalamus. In Chapter-3, we discovered that also in the SCN different cell types are selectively changed in T2DM subjects. These changes included a decrease of the number of arginine vasopressin (AVP) expressing neurons and glial fibrillary acidic protein (GFAP) expressing astroglial cells, a loss of daily rhythmicity in the AVP expressing neurons, and a loss of seasonal rhythmicity in the ionized calcium-binding adapter molecule 1 (Iba1) expressing microglial cells. Since all these cell populations are important for an optimal functioning of the central brain clock, these data provide the first evidence that chronic T2DM pathogenesis is substantially linked to a dysfunction of the biological clock machinery in humans. Consequently, these results also suggest that for an effective treatment of T2DM, it may be useful to consider lifestyle
changes and/or medication that also targets the biological clock. T2DM is also known to increase the risk of Alzheimer’s disease (AD), whereas proper anti-diabetic treatment attenuates AD development. In Chapter-4, we studied the association between T2DM and cholinergic neurons in the human nucleus basalis of Meynert (NBM). As shown previously, we also found that NBM cholinergic neurons were relatively stable in number during the course of AD and only declined at later stages of the disease, while the choline acetyltransferase (ChAT) expression already declined earlier. Tau pathology showed a positive correlation with AD progression. T2DM subjects did not show a decrease in the number or ChAT expression of the cholinergic neurons, nor an increased tau pathology as compared to non-diabetic controls. However, these pathological changes were significantly reduced when patients were treated with insulin. These data emphasize the role of impaired insulin signaling in AD development and, maybe even more important, it implies that insulin replacement may be effective in lowering the progression of this disease.

Chapter-5 summarizes the knowledge on how cellular energy metabolism is integrated in microglial functionality. Glucose is vital for microglia survival and several glucose transporters are expressed to ensure sufficient glucose influx. Fatty acids are another source of energy for microglia and in fact have been shown to influence microglial activity mostly. Glutamine is also used as an energy substrate by microglia, but has been shown to have neurotoxic effects when overloaded. Fuel influx in microglia is tightly associated to microglial reactivity under different pathophysiological conditions and is thought to be the underlying cause of hypothalamic dysfunction associated with obesity. It is known that hypercaloric diets lead to microglial activation. However, little is known about daily rhythmicity in microglial immune and metabolic functions, either in lean or obese conditions. Therefore, in Chapter-6, we studied how microglial immunometabolism is disturbed by hypercaloric diets. We observed that the significant rhythmicity of circadian clock genes in microglia under chow conditions, were partially lost during diet-induced obesity (DIO). The expression of microglial genes involved in immune responses also showed significant time-of-day differences, which were disrupted in HFD-fed animals. As indicated by gene expression evaluation microglia responded to the obesogenic conditions by a shift of substrate utilization with a decrease in the animals active period of glutamate and glucose metabolism, and an overall increase of lipid metabolism. Finally, evaluation of monocyte gene expression only showed small or absent effects of HFD on these peripheral myeloid cells, suggesting a cell-specific microglial inflammatory response due to DIO.
Samenvatting

Het voornaamste doel van dit proefschrift was het onderzoeken van de neuronen en gliacellen in de voorhersenen van mensen met een metabole verstoring, met name obesitas en (pre) diabetes. In Hoofdstuk-1 geven we een overzicht van de peptiderge neuronen en gliacellen in de infundibulaire kern (IFN) van de mediobasale hypothalamus in de context van overeten, overgewicht en diabetes. Dit hersengebied is het belangrijkste hypothalame gebied voor de controle van voedselinname en lichaamsgewicht. We hebben ons met name gericht op de perifere hormonen, en nutriënten die betrokken zijn bij de regulatie van de energiehomeostase, zoals leptine, insuline, glucose en vetzuren, en hoe deze inwerken op de peptiderge neuronen en gliacellen van de IFN. Daarnaast hebben we de belangrijkste intra-hypothalame projecties van de IFN beschreven.

In de volgende experimentele hoofdstukken hebben we onderzocht hoe de neuronen en gliacellen in verschillende gebieden van het humane brein die betrokken zijn bij de controle van het energie metabolisme (hoofdstuk-2), het circadiane ritme (hoofdstuk-3) en geheugen en cognitie (hoofdstuk-4) beïnvloedt worden door T2DM. In Hoofdstuk-2 hebben we in post-mortem hypothalami, de IFN van patiënten met T2DM vergeleken met de IFN van een gepaarde groep controle personen zonder T2DM. In deze studie hebben we ook het effect van de verschillende anti-diabetische behandelingen onderzocht. Voor deze vergelijking heb ik de proopiomelanocortine (POMC) en neuropeptide-Y (NPY) immunoreactieve (-ir) neuronen en de geïoniseerde calcium-bindende adapter molecuul 1 (iba1)-ir en transmembraan proteïne 119 (TMEM119)-ir microglia in de IFN gekarakteriseerd. Vergeleken met de controlepersonen hadden de T2DM patiënten significant lager aantal POMC-ir neuronen, maar geen veranderingen in de NPY-ir neuronen of microglia. Interessant is dat de T2DM patiënten die met het anti-diabetische middel Metformine behandeld waren minder NPY-ir neuronen en minder microglia hadden in vergelijking met T2DM patiënten zonder Metformine behandeling. Verder was het aantal microglia gecorreleerd met het aantal NPY-ir neuronen, maar alleen bij T2DM patiënten. Deze resultaten geven aan dat T2DM gepaard gaat met verschillende veranderingen in POMC- en NPY-neuronen en microglia in het IFN. Bovendien zijn de verschillende T2DM-behandelingen geassocieerd met zeer selectieve veranderingen in hypothalame neuronen en microglia.

Het is bekend dat de IFN in het achterste deel van de hypothalamus een reciprope interactie heeft met de suprachiasmatische kern (SCN), de kern waar de centrale biologische klok zich bevindt, in het voorste deel van de hypothalamus. In Hoofdstuk-3 beschrijven we dat verschillende soorten cellen in de SCN selectief zijn veranderd in patiënten met T2DM in vergelijking met de niet-T2DM personen. Deze veranderingen behelzen voornamelijk een afname van het aantal arginine vasopressine (AVP)-ir neuron en glial fibrillary acidic protein (GFAP)-ir astrocyten, verlies van de dagelijkse ritmiek in de AVP-ir neuron en verlies van het seizoensgebonden
ritme in iba1-ir microglia. Aangezien al deze cel populaties een belangrijke rol spelen bij het in stand houden van een goed functionerende biologische klok, bewijzen deze bevindingen voor het eerst dat chronische T2DM verbonden is met een disfunctioneren van de centrale brein klok in de SCN. Deze resultaten suggereren ook dat levensstijlveranderingen en/of medicatie gericht op de biologische klok een aanvullend effect zouden kunnen hebben bij de reguliere behandeling van T2DM.

Het is reeds langer bekend dat T2DM het risico op de ziekte van Alzheimer (AD) verhoogt en dat een goede anti-diabetische behandeling de ontwikkeling van AD vertraagt. In Hoofdstuk-4 hebben we de associatie tussen T2DM en de cholinerge neuronen in de humane nucleus basalis van Meynert (NBM) bestudeerd. We vonden dat het aantal cholinerge neuronen in de NBM relatief stabiel bleef tijdens de progressie van AD en dat deze pas in een laat stadium afnam. In tegenstelling tot het aantal neuronen, nam de expressie van het enzym choline acetylntransferase (ChAT) in deze neuronen al in een vroeg stadium af. De ophoping van Tau eiwitten bleek daarentegen toe te nemen naarmate de progressie van AD verder vorderde. Hoewel deze parameters geen verschil lieten zien tussen T2DM patiënten en niet-T2DM personen, zagen wij wel significant minder pathologische veranderingen in de T2DM patiënten die behandeld waren met insuline in vergelijking met de T2DM patiënten die geen insuline hadden ontvangen. Deze bevindingen benadrukken de rol van verminderde insuline gevoeligheid tijdens de ontwikkeling van AD. Daarnaast impliceren deze resultaten dat insuline therapie de progressie van deze ziekte kan vertragen.

**Hoofdstuk-5** is gericht op het samenvatten van de kennis over de integratie tussen het cellulaire energiemetabolisme en de functionaliteit van microglia. Glucose is van vitaal belang voor het functioneren van microglia en microglia hebben dan ook verschillende glucosetransporters om voldoende glucose op te kunnen nemen. Vetzuren zijn een andere energiebron voor microglia en er is aangetoond dat vooral deze vetzuren de activiteit van microglia beïnvloeden. Glutamine kan ook door microglia worden gebruikt als een energiesubstraat, echter blijkt dit bij overbelasting neurotoxische effecten te hebben. De brandstoffinvoer in microglia is nauw verbonden met de reactiviteit van microglia onder verschillende pathofysiologische omstandigheden en er wordt verondersteld dat dit een onderliggende oorzaak is van de hypothalamische dysfunctie tijdens obesitas. Het is bekend dat hypercalorische diëten resulteren in activatie van microglia. Er is echter weinig bekend over een mogelijk dagelijks ritme in de immuun- en metabolische functies van microglia, in gezonde dan wel metabool verstoorde omstandigheden. Daarom hebben we in **Hoofdstuk-6** onderzocht hoe het immuun metabolisme van microglia wordt verstoord door een hypercalorisch diëet. De significante dagelijks ritmiek van de circadiane klokgenen in microglia in controle condities ging gedeeltelijk verloren in condities van dieet-geïnduceerde obesitas (DIO). Ook de expressie van immuun gerelateerde genen vertoonde duidelijke dag/nacht verschillen in microglia, ook deze ritmiek was verstoord in dieren gevoed met een hoog-vet dieet (HFD). Evaluatie van de genexpressie gaf aan dat microglia reageerden op de
obesogene omstandigheden door een verschuiving van het substraatgebruik. De microglia lieten een afgenomen metabolisme van glutamaat en glucose zien tijdens de actieve periode van de dieren in combinatie met een algemene toename van het lipidenmetabolisme. Bovendien suggereren de gegevens over de bio-energetica en de dynamiek van mitochondriën dat de energieproductie in microglia verhoogd was tijdens de inactieve periode van HFD dieren. Ten slotte toonde een evaluatie van de genexpressie van monocyten slechts een klein of afwezig effect van HFD aan op deze perifere myeloïde cellen, wat suggereert dat de ontstekingsreactie in DIO cel specifiek is voor microglia.
List of publications


1. PhD training

General courses
- “Animal course” (Art.9), Amsterdam, NL, 2016
- “AMC world of science”, Amsterdam, NL, 2016
- “Weekly Research meeting Endocrinology”, Amsterdam, NL, 2015-2020
- “Weekly NIN neuroscience symposium”, Amsterdam, NL, 2017-2020

Oral presentations
- “Amsterdam Gastroenterology & Metabolism (AG&M) PhD-students retreat”, Lunteren, NL, 2016
- “Jonge NVDO bijeenkomst”, Soesterberg NL, 2017
- “Amsterdam Gastroenterology & Metabolism (AG&M), garderen, 2017
- “Annual Dutch Diabetes Research Meeting” Oosterbeek, NL, 2017
- “North Europe Young Diabetologists (NEYD) Annual Meeting” Warwickshire, UK, 2017
- “Amsterdam Gastro-enterology & Metabolism” Garderen, 2018
- “GliaNED meeting” Rotterdam, NL, 2018
- “Dutch Neuroscience meeting”, Lunteren, NL, 2018
- “Annual Dutch Diabetes Research Meeting” Oosterbeek, NL, 2018
- “Annual Dutch Diabetes Research Meeting” Oosterbeek, NL, 2019

Poster presentations
- “Society for Neuroscience” San Diego, USA, 2018
- “Muscle Clocks and Diabetes symposium” Amsterdam, NL, 2019

Meetings
- “Dutch Neuroscience meeting” Lunteren, 2016
- “Neurex, the arcuate nucleus: sensor of time and metabolism” Strasbourg, FR, 2018
- “Dutch Neuroscience meeting” Lunteren, 2017

2. Teaching
- Samantha Wolff (MSc internship, 6 months)
- Laurie Mulder (MSc thesis, 5 months)
- Rick Hoogenboom (BSc internshio, 6 months)
- Marit Koenen (BSc internship, 6 months)
- Felipe Corea da Silva (MSc internship, 6 months)
- Thuc-Anh Nguyen (MSc internship, 6 months)
About the author

Finally the moment is here, Martin has finished his PhD.

It all started on 5 September 1982 when Marten Jan Thomas was born in Rottevalle, a small village near Drachten, in the southeast of Friesland. Two years later Martin moved with his parents and elder sister to Amsterdam. After he finished high school (Pieter Nieuwland College) in 1999, it took him several years as well as several studies to figure out what would be the perfect match for him. He started close to home with studying mechanical engineering, but after 1 year he decided this was not the right choice and took a different approach. In the new millennium he went to Utrecht to study Life Science. However, also this time he was not convinced this was the best study for him and thus decided to try an academic career in Medicine at the AMC.

When almost halfway he realized again this was not it and decided to zoom out and overthink his choices. He tried completely different careers this time, such as being a bartender and becoming a jet fighter pilot.

After a couple of years he realized there’s more than one way to skin a cat and picked up his Life Science study in Utrecht again. After obtaining his bachelor degree in 2012 he started a master in Biomolecular Sciences at the VU. Before he started his PhD research at the AMC-NIN, he got married and became a father. Now in 2020, almost 5 years later and in the meanwhile father of both a son and a daughter, we are here; after some years of learning and wandering he finished his PhD. As maybe clear from the foregoing the next step is not clear yet, but can be many fold.
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Everybody else from the Yi group thanks for your scientific input during the Yi-meetings. Thanks, Yuanqing for helping me with PCR, Xiaolan for showing me around Strasbourg, Fernando for being the best cricket player that I know, Clarissa for your beautiful pictures of lipid droplets and Brazilian doors. And last but not least, Irina, thanks for being the awesome person that you are. Whenever I see a pizza being prepared in a frying pan, I will think of you and Nikita.

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