REVIEW

The role of orexin in controlling the activity of the adipo-pancreatic axis

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Abstract

Orexin A and B are two neuropeptides, which regulate a variety of physiological functions by interacting with central nervous system and peripheral tissues. Biological effects of orexins are mediated through two G-protein-coupled receptors (OXR1 and OXR2). In addition to their strong influence on the sleep–wake cycle, there is growing evidence that orexins regulate body weight, glucose homeostasis and insulin sensitivity. Furthermore, orexins promote energy expenditure and protect against obesity by interacting with brown adipocytes. Fat tissue and the endocrine pancreas play pivotal roles in maintaining energy homeostasis. Since both organs are crucially important in the context of pathophysiology of obesity and diabetes, we summarize the current knowledge regarding the role of orexins and their receptors in controlling adipocytes as well as the endocrine pancreatic functions. Particularly, we discuss studies evaluating the effects of orexins in controlling brown and white adipocytes as well as pancreatic alpha and beta cell functions.

Key Words

- ▶ adipocyte
- ▶ adipose tissue
- ▶ islet cells
- ▶ orexin

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Introduction

The neuropeptides termed as orexin A and B (hypocretin 1 and 2) were originally identified in rat hypothalamus as products of proteolytic cleavage of the precursor protein – preproorexin (de Lecea *et al.* 1998, Sakurai *et al.* 1998). Orexin A and orexin B are composed of 33 and 28 amino acids, respectively. Both peptides bind to two G-protein-coupled receptors termed as orexin receptor 1 (OXR1) and orexin receptor 2 (OXR2). OXR1 is equipotently activated by both peptides, whereas OXR2 has approximately ten-fold selectivity for orexin B (Smart *et al.* 1999). Orexin receptor signaling is multifaceted; nevertheless, MAP kinases, adenylyl cyclase, calcium ions and phospholipases are considered as essential intracellular molecules transmitting the signals upon orexin receptors activation (Kukkonen & Leonard 2014, Kukkonen 2014). Early reports

indicated the importance of orexins and their receptors in controlling body weight and energy homeostasis by stimulating food intake, energy expenditure (Lubkin & Stricker-Krongrad 1998, Sakurai *et al.* 1998) as well as by controlling sleep–waking cycle (Lin *et al.* 1999). Animal studies in mice and dogs provided evidence that orexin deficiency or genetic inactivation of OXR2 cause narcolepsy (Chemelli *et al.* 1999, Lin *et al.* 1999). Furthermore, orexin A deficiency in humans is associated with narcolepsy, an excessive daytime sleepiness (Nishino *et al.* 2000). Since narcolepsy is associated with a higher risk of obesity and type 2 diabetes mellitus (Honda *et al.* 1986), it was assumed that orexin deficiency may contribute to glucose disbalance and pathophysiology of obesity (Schuld *et al.* 2000). Indeed, transgenic mice

in which orexin-containing neurons are ablated, develop narcolepsy as well as obesity (Hara *et al.* 2001). In contrast, orexin or OX2R overexpression in rodents protects from diet-induced obesity, improves glucose control as well as leptin sensitivity (Funato *et al.* 2009). It is important to acknowledge that the lack of leptin or its insensitivity are linked to pathophysiology of obesity and insulin resistance (Ingalls *et al.* 1950, Hummel *et al.* 1966, Girard 1997). Exogenous orexin A attenuates adiposity in rats and mice with diet-induced obesity, further supporting the observations in genetically modified animals (Novak & Levine 2009, Perez-Leighton *et al.* 2012, 2013).

Recently, mechanisms of action through which orexin A affects energy homeostasis, glucose control and body mass were reported. Injection of orexin A into the ventromedial hypothalamus activates sympathetic nervous system, thereby promoting glucose uptake and glycogen synthesis in the skeletal muscle (Shiuchi *et al.* 2009). Furthermore, orexin is required to maintain motor activity (Hara *et al.* 2001) as well hypothalamic insulin signaling, which results in improvement of peripheral insulin sensitivity (Tsuneki *et al.* 2008).

Maintenance of body weight and energy homeostasis is precisely controlled by orchestration of multiple metabolic and hormonal factors released from peripheral tissues. Outside of the central nervous system, orexins and/or their receptors are expressed in the gastrointestinal tract, reproductive system, adrenal glands, heart, pancreas and adipose tissue in rodents as well as in humans (Johren *et al.* 2001, Nakabayashi *et al.* 2003, Digby *et al.* 2006, Skrzypski *et al.* 2011, Shen *et al.* 2013).

Orexin A circulates in blood and its levels are negatively correlated with BMI in humans (Adam *et al.* 2002, Tomasik *et al.* 2004, Baranowska *et al.* 2005). Plasma orexin A levels are negatively associated with insulin resistance and positively correlated with insulin sensitivity in type 2 diabetic patients (Zarifkar *et al.* 2017). Furthermore, plasma orexin levels are low in pregnant women who suffer from gestational diabetes mellitus (Yilmaz *et al.* 2013). These data suggest a functional relevance of orexin in the context of pathophysiology of obesity and type 2 diabetes.

It is well known that body weight as well as glucose control and whole body metabolism are influenced by adipose tissue, pancreatic alpha and beta cell functions and *vice versa*. Adipose tissue not only stores energy, however, it also produces and releases a large number of factors that participate in the regulation of energy homeostasis (Rosen & Spiegelman 2006). Adipose tissue dysfunction is not only a hallmark of obesity; however,

it strongly increases insulin resistance in type 2 diabetes mellitus (Frayn *et al.* 2007). Pancreatic alpha and beta cells produce and release glucagon and insulin, respectively. Insulin decreases postprandial blood glucose levels, whereas glucagon raises glucose in bloodstream (Grossman 1986). The loss of pancreatic beta cells and dysregulation of insulin and glucagon secretion contribute to impaired glucose control in type 1 and type 2 diabetes (Maedler & Donath 2004, D'Alessio 2011).

In summary, orexin interacts with adipose tissue and with the endocrine pancreas. Orexin is linked to the pathophysiology of obesity and diabetes. Taking this into consideration, we summarize the recent studies evaluating the importance of orexins in controlling glucose and lipid metabolism.

Orexin in adipose tissue

Expression of orexin and orexin receptors in white adipose tissue

Despite some inconsistencies, it appears that both OX1 and OX2R were identified in adipocytes. While OX1 and OX2R were identified in adipocytes isolated from subcutaneous and omental human fat tissue (Digby *et al.* 2006), others found that OX1 but not OX2R is present in human preadipocytes (Pino *et al.* 2017). Mature adipocytes isolated from abdominal subcutaneous (sc-ab), neck subcutaneous (sc-nk) and deep neck (dp-nk) regions fail to express orexin receptors (Pino *et al.* 2017). However, it must be noted that the study by Digby *et al.* utilized adipose tissue obtained from patients undergoing elective surgery (Digby *et al.* 2006). In the study using differentiated adipocytes obtained from patients (sc-nk, dp-nk) as well as healthy individuals (sc-ab) heterogeneous patients were included. Therefore, the discrepancies regarding the expression of orexin receptors may be due to the inclusion of heterogeneous study populations. Since both studies used RT-PCR, conventional (Digby *et al.* 2006) vs quantitative (Pino *et al.* 2017), the discrepancies are rather not explained by the methodological differences. Sensitivity of both methods is comparable (Bastien *et al.* 2008).

We identified both *Oxr1* and *Oxr2* mRNA expression in rat adipocytes as well as in both, non-differentiated and fully differentiated 3T3-L1 cells (mouse embryonic fibroblast – adipocyte-like cell line) (Skrzypski *et al.* 2011). mRNA expression and protein production of OX1 in rat adipocytes was reported by Shen *et al.* (2013). Of note,

the authors described reduced *OXR1* protein production in obese rats as compared to non-obese animals (Shen *et al.* 2013). *OXR1* and *OXR2* mRNA are also expressed in porcine preadipocytes (Wojciechowicz *et al.* 2016). Furthermore, both orexin receptors are present in porcine subcutaneous and visceral adipose tissue as well as in mature porcine visceral adipocytes on mRNA and protein levels. Noteworthy, the expression of *OXR1* is higher compared to *OXR2* in both investigated fat depots and isolated adipocytes (Pruszyńska-Oszmalek *et al.* 2018).

Preproorexin mRNA is expressed in white adipose tissue in mice (Zhang *et al.* 2010b). However, in this study, animals were fed diet containing lard (5 or 45%); therefore, it cannot be excluded that this diet could contribute to induction of preproorexin mRNA in adipose tissue. In support of this notion, mice fed high-lard diet (45%) displayed approximately two-fold higher expression of preproorexin, compared to animals fed chow diet supplemented with 5% lard. Stimulation of preproorexin mRNA expression by high-fat diet is supported by the results of a study performed in rats (Barson *et al.* 2012). Barson and colleagues showed that lard (41%) diet upregulates preproorexin expression in the perifornical lateral hypothalamus.

There is evidence indicating that lard oil induces endoplasmic reticulum (ER) stress in rat adipose tissue (Nivala *et al.* 2013). Importantly, ER stress stimulates the activity of numerous transcription factors (Jiang *et al.* 2016), which suggests that ER may be relevant in conferring lard diet-induced preproorexin expression. Nevertheless, due to heterogenic nature of adipose tissue, which is composed of many cell types including preadipocytes adipocytes, macrophages and fibroblasts (Altintas *et al.* 2011), the cellular source of preproorexin mRNA in fat tissue is unknown. Studies utilizing isolated murine adipocytes should answer the question whether preproorexin is present in adipocytes. By contrast, preproorexin mRNA is absent in human adipose tissue (Digby *et al.* 2006). Overall, the majority of studies showed that white rodent and porcine adipocytes express both isoforms of orexin receptors. By contrast, expression of orexin receptors in human mature adipocytes requires further investigations.

The role of orexins in white preadipocytes

Using 3T3-L1 preadipocytes, we and others reported that orexin A stimulates, whereas orexin B suppresses preadipocytes proliferation *in vitro* (Zwirski-Korczala *et al.*

2007, Skrzypski *et al.* 2012). Orexin A can also enhance proliferation of rat primary preadipocytes as well as NIH 3T3 cells. Both orexin A and B can augment porcine preadipocytes growth (Wojciechowicz *et al.* 2016). Orexin A protects 3T3-L1 preadipocytes from apoptosis induced either by serum deprivation or by palmitic acid administration (Skrzypski *et al.* 2012). The promitogenic and antiapoptotic properties of orexin A (Fig. 1) are mediated via ERK1/2-dependent mechanism (Skrzypski *et al.* 2012).

The role of orexins in controlling rodent preadipocytes differentiation into mature adipocytes is not completely understood. While *Oxr1* mRNA expression increases during differentiation of 3T3-L1 preadipocytes (Skrzypski *et al.* 2011), orexin A fails to affect differentiation of rat or 3T3-L1 preadipocytes (Skrzypski *et al.* 2012). However, a recent study showed that orexin A has ability to increase *Pparg2* expression in undifferentiated 3T3-L1 cells *in vitro* (Shen *et al.* 2013). PPARG2 plays a prominent role in inducing adipogenesis (Tontonoz *et al.* 1994a,b); therefore, the influence of orexin A on rodent preadipocytes differentiation needs to be studied in a more detailed fashion.

In contrast to rodents, we recently showed that both orexin A and B can enhance isolated porcine preadipocytes differentiation as judged by the increased lipid accumulation and the expression of proadipogenic genes: *PPARG2*, *C/EBPA* and *C/EBPB* (Wojciechowicz *et al.* 2016). Studies evaluating the role of orexins in the context of human adipogenesis are scarce. To our knowledge, there is only one *in vitro* study available to date that reported an inability of orexin A to induce the expression of adipogenic markers in preadipocytes isolated from sc-ab and sc-nk adipose tissue (Pino *et al.* 2017). Overall, orexins may stimulate or suppress preadipocytes proliferation and differentiation in a species-specific manner. While in rodents, orexin A stimulates preadipocytes growth and protects them from apoptosis, orexin B suppresses cell proliferation. By contrast, orexins A and B induce proliferation and differentiation of preadipocytes isolated from pigs. In humans, however, orexin A rather fails to stimulate the differentiation of preadipocytes.

The role of orexins in regulating mature white adipocytes functions and adipokines production

Orexin A but not orexin B reduces glycerol release and the expression of hormone-sensitive lipase (*HSL*) in human

Figure 1

Summary of the orexin A (OXA) effects on preadipocytes and mature adipocytes of white adipose tissue, brown adipose tissue and endocrine cells of pancreatic islets. A full color version of this figure is available at <https://doi.org/10.1530/JOE-18-0122>.

omental adipose tissue *in vitro* (Digby *et al.* 2006). Orexin A stimulates free fatty acid synthesis in freshly isolated rat adipocytes and increases intracellular triacylglycerol content in 3T3-L1 adipocytes. Furthermore, orexin A suppresses glycerol release as well as *Hsl* mRNA expression in 3T3-L1 adipocytes (Skrzypski *et al.* 2011), which is consistent with findings reported in humans (Digby *et al.* 2006). Suppression of lipolysis by orexin A but not orexin B was also reported in isolated porcine adipocytes (Pruszyńska-Oszmalek *et al.* 2018). Overall, these *in vitro* results suggest that orexin A exerts its lipogenic and antilipolytic activities by acting directly on adipocytes. Nevertheless, it must be noticed that recent human studies revealed that orexin A has no effects on lipolysis in fat tissue explants derived from the neck or abdominal regions (Pino *et al.* 2017). As discussed earlier, the same study also showed that differentiated adipocytes lack orexin receptors, which may explain the lack of effects of orexin A on lipolysis in fat tissue explants. Thus, orexin A may affect lipid metabolism in rodents and pigs; however, there is a lack of convincing evidences in humans.

Our laboratory provided evidence that activation of orexin receptors results in increased rate of glucose uptake in differentiated 3T3-L1 mature adipocytes and isolated rat primary adipocytes. This process requires activation of PI3-K/PKB pathway that in turn leads to

enhanced translocation of the glucose transporter GLUT4 to the plasma membrane (Skrzypski *et al.* 2011). Orexin A enhances glucose uptake by stimulating GLUT4 expression in porcine adipocytes (Pruszyńska-Oszmalek *et al.* 2018). GLUT4 plays a prominent role in insulin-induced glucose uptake and its selective depletion in adipose tissue causes insulin resistance (Abel *et al.* 2001).

A recent study reported that orexin A has the ability to increase *Glut4* mRNA expression in 3T3-L1 adipocytes (Shen *et al.* 2013), concurring our findings. Stimulation of *Glut4* mRNA expression by orexin A was attenuated by pharmacological blockade of MAP kinases members (ERK1/2, JNK, p38) or OXR1 (Shen *et al.* 2013). Overall, these data suggest that orexin A may stimulate glucose uptake in adipocytes by inducing GLUT4 translocation into the plasma membrane via PI3-K/PKB-dependent mechanism.

Human and rodent data show that orexin A increases PPARG2 expression in adipocytes (Digby *et al.* 2006, Skrzypski *et al.* 2011, Shen *et al.* 2013). The lipogenic potency of orexin A can be attenuated by blocking PPARG expression and activity. However, Shen *et al.* found that orexin A can activate multiple MAP kinases in 3T3-L1 adipocytes and their inhibition was sufficient to block orexin A-induced lipogenesis, *Glut4* and *Pparg2* expression (Shen *et al.* 2013). Taken together, PPARG and MAP kinases are required for orexin A-induced lipogenesis.

Adipocytes produce and release a variety of hormones. Among them are adipokines that strongly influence glucose metabolism, insulin sensitivity and play a role in the pathophysiology of obesity (Ouchi *et al.* 2011). We reported that orexin A can stimulate expression and secretion of adiponectin in 3T3-L1 adipocytes (Skrzypski *et al.* 2011). Furthermore, orexin A upregulated plasma concentrations of adiponectin in lean, obese and T2DM animals, and increased FGF-21 levels in animals with T2DM (Kaczmarek *et al.* 2017). Notably, there is convincing evidence indicating that adiponectin promotes insulin sensitivity (Yamauchi *et al.* 2001, Awazawa *et al.* 2011) and the lack or low levels of adiponectin are relevant in the context of pathophysiology of T2DM (Lindsay *et al.* 2002). *In vivo*, orexin A can reduce leptin levels in rats and mice (Switonska *et al.* 2002, Park *et al.* 2015). By contrast orexin A stimulates leptin secretion and expression in porcine-isolated adipocytes (Pruszyńska-Oszmalek *et al.* 2018).

In a rat model of T2DM and obesity, we reported that treatment with orexin A for 4 weeks decreases fasting levels of glucose in these animals (Kaczmarek *et al.* 2017). Furthermore, orexin A-treated T2DM rats had improved glucose tolerance. Obese and T2DM animals treated with orexin A had reduced plasma levels of proinflammatory cytokines TNF- α , resistin and visfatin (Kaczmarek *et al.* 2017). These three cytokines contribute to insulin resistance, impair insulin secretion and play a role in pathophysiology of obesity (Stofkova 2010, Makki *et al.* 2013).

Since all of these hormones are produced and secreted by adipocytes (Arita *et al.* 1999, Muise *et al.* 2008), it is possible that orexin A can improve glucose control and insulin sensitivity by influencing the release of these pro- and anti-inflammatory adipokines in a favorable manner. However, it remains not clear whether orexin A modulates adipokines levels directly or by normalizing other metabolic parameters such as blood glucose or body weight. Notably, reduction of body weight is paralleled by the reduction of circulating proinflammatory cytokines in humans and animals (Ziccardi *et al.* 2002, Rivera *et al.* 2008, Tajik *et al.* 2013). On the other hand, as already discussed before, orexin A stimulates adiponectin expression and secretion *in vitro*. Therefore, modulation of pro- and/or anti-inflammatory cytokines may be complex and encompass direct and indirect interaction with several target tissues such as adipocytes. Through these multiple interactions, orexin may attenuate metabolic diseases including obesity.

Overall, a significant part of data regarding the function and expression of orexin receptors in adipose tissue

came from *in vitro* studies; therefore, the physiological relevance of these findings needs to be confirmed *in vivo*. Since enhanced orexin 2 receptor signaling or orexin overexpression are associated with reduced body weight in animals with diet-induced obesity (Funato *et al.* 2009), lipogenic activities of orexin reported in *in vitro* studies are rather not relevant *in vivo*. It appears that the direct effects of orexin A on adipocytes are overridden by the possible interaction with antilipogenic factors *in vivo*. For example, orexin A can rise corticosterone levels *in vivo* (Malendowicz *et al.* 1999). Glucocorticoids can stimulate lipolysis in adipocytes (Xu *et al.* 2009). In addition, orexin potentiates physical activity and energy expenditure (Lubkin & Stricker-Krongrad 1998, Perez-Leighton *et al.* 2012), which may counteract orexin A-induced lipid accumulation.

On the other hand, it appears likely that orexin A contributes to changes of the morphology of white fat tissue via stimulation of preadipocytes proliferation and through inhibition of apoptosis. Of central importance in this context is the transcriptional receptor factor PPARG, which has the ability to increase insulin sensitivity and to enhance glucose uptake and adiponectin expression (Maeda *et al.* 2001, Liao *et al.* 2007, Sharma & Staels 2007).

Activation of PPARG leads to increased number of small preadipocytes. These small adipocytes are, in contrast to large adipocytes, more insulin sensitive (de Souza *et al.* 2001). Newly differentiated small adipocytes have a potent storage capacity for lipids. This ability of small adipocytes is responsible for reduction of circulating FFA. It is well known that FFA deteriorates insulin sensitivity by several mechanisms: inhibition of insulin-stimulated glucose uptake, suppression of glycogen synthase activity (Boden 1999) and promotion of inflammation (Sears & Perry 2015). Therefore, reduction of circulating FFA contributes to improved insulin sensitivity in obesity and T2DM (Daniele *et al.* 2014).

Orexin and brown adipose tissue

In contrast to white adipocytes, which serve mainly as energy storage in addition to producing of cytokines, brown fat cells are able to generate heat (Smith & Horwitz 1969). Initially, it was thought that the presence of BAT is restricted to the newborns only; however, later studies showed that BAT is present in adult humans and of the amounts of BAT are inversely correlated with BMI (Cypess *et al.* 2009). Activation of brown adipose tissue promotes oxidative metabolism and energy expenditure

(Ouellet *et al.* 2012). These processes are conferred via UCP1 protein (Nicholls *et al.* 1978). The capacity of brown adipocytes to generate heat is precisely controlled by orchestration of environmental and hormonal factors as well as the sympathetic nervous system (Richard *et al.* 2010). Orexin receptors are highly expressed in brain regions responsible for thermoregulations such as *raphe pallidus* (Marcus *et al.* 2001). Furthermore, it was found that cold stress leads to stimulation of preproorexin mRNA expression in lateral hypothalamic area (Ida *et al.* 2000), indicating that orexin may be involved in controlling body temperature. Concordantly, it was found administration of orexin A into the lateral cerebral ventricle stimulates sympathetic firing rate and increases colonic temperature (Monda *et al.* 2001). In line with these results, injection of orexin A into the rostral *raphe pallidus* induces thermogenesis in BAT (Tupone *et al.* 2011). Elevation of body temperature was also detected after orexin A administration into the fourth ventricle in rats (Zheng *et al.* 2005). Another study found that mice with neuron-specific orexin deficiency have attenuated stress-induced hyperthermia and reduced UCP1 expression in BAT (Zhang *et al.* 2010a). In mice, orexin neurons are also required for prostaglandin E2-induced fever as well as for the adaptation to environmental cooling (Takahashi *et al.* 2013). In line with these results, ablation of hypothalamic orexin neurons results in reduced BAT thermogenesis (Mohammed *et al.* 2014). Others reported that central administration of orexin A promotes thermogenesis; however, these effects are not mediated via changes in UCP1 expression in BAT (Yoshimichi *et al.* 2001, Russell *et al.* 2002). Another study showed that orexin is involved in conferring thermogenic effects of bone morphogenetic protein 8B (BMP8B) (Martins *et al.* 2016). This study found that intracerebroventricular injection of BMP8B induces thermogenesis and UCP1 expression in BAT, which is associated with increased preproorexin mRNA expression in the lateral hypothalamic area in rats. These effects were reversed in mice lacking orexin or OX1 (Martins *et al.* 2016).

In contrast to thermogenic properties of orexin A, it was found that pharmacological blockade of OX1 receptor by the antagonist SB-334867-A leads to increased thermogenesis via upregulation of *Ucp1* in BAT in *ob/ob* mice (Haynes *et al.* 2002). Furthermore, a recent study demonstrated that orexin-deficient mice have higher amounts of BAT deposits (Ramanathan & Siegel 2014). Results in experiments with orexin-null mice with diet-induced obesity suggest that orexin A is anti-adipogenic (Sellayah *et al.* 2011). Increased body

weight in mice lacking orexin was associated with enhanced metabolic efficiency but not with increased food consumption. Additionally, orexin-depleted obese mice had reduced energy expenditure and decreased O₂ consumption. In BAT isolated from orexin A-null mice, several thermogenic genes such as *Pgc-1a*, *Tfam* and *Ucp1* were downregulated when compared to the levels detected in BAT obtained from WT animals. Furthermore, mice with orexin deficiency showed reduced *Pparg* expression and triacylglycerol content in BAT, suggesting that orexin A stimulates the differentiation of brown preadipocytes. The relevance of orexin in regulating brown adipogenesis was shown in studies utilizing orexin-deficient mice. These animals had lipodystrophy of intrascapular BAT. Direct positive effects of orexin on brown preadipocytes differentiation were confirmed by *in vitro* experiments showing the ability of orexin to induce differentiation of mesenchymal C3H10T1/2 cells, HIB1B cells (brown preadipocytes cell line) as well as primary murine brown preadipocytes (Sellayah *et al.* 2011). Mechanisms of orexin-enhanced brown preadipocytes differentiation include OX1-mediated phospholipase C and p38 MAP kinase activation (Sellayah *et al.* 2011, Sellayah & Sikder 2012). In summary, orexin A is required for adipogenesis of BAT in rodents. Furthermore, these results allow claiming that the loss of BAT in orexin-depleted mice may be a link between orexin deficiency and obesity. In agreement with this hypothesis, application of orexin can reverse BAT dysfunction caused by aging in rodents, thereby leading to improved glucose control and body weight loss (Sellayah & Sikder 2013). By contrast, there is evidence indicating that orexin A fails to stimulate the differentiation of brown preadipocytes as well as the expression of thermogenic genes in preadipocytes and differentiated brown fat cells obtained from human individuals (Pino *et al.* 2017). Therefore, thermogenic properties of orexin A could be also species specific.

Orexin in the endocrine pancreas

Expression of orexins and orexin receptors in endocrine cells of pancreatic islets

A vast part of experiments evaluating the presence of orexin system in endocrine pancreas was performed using isolated rat pancreatic islets. *Oxr1* and *Oxr2* mRNAs are present in rat pancreatic islets (Nowak *et al.* 2005). On the protein level, the presence of OX1, but not OX2, was reported in protein lysates derived from rat and murine

islets (Gonc^z *et al.* 2008, Park *et al.* 2015). Thus, expression of OXR2 in pancreatic islets may be restricted to mRNA level, only.

Double immunofluorescence staining revealed that OXR1 is present in glucagon- and insulin-immunoreactive cells in rats (Ouedraogo *et al.* 2003, Gonc^z *et al.* 2008, Adeghate *et al.* 2010). In agreement with these findings, OXR1 protein production was detected in rat insulinoma INS-1 and hamster glucagonoma InR-G9 cell lines (Gonc^z *et al.* 2008, Chen *et al.* 2013). It is important to note that OXR1 was also detected in intrapancreatic nerves in rats and guinea pigs (Kirchgessner & Liu 1999, Adeghate *et al.* 2010). The complexity of orexin system in pancreatic islets is supported by studies showing the immunoreactivity of orexin A in endocrine cells of islet. Orexin A was found in pancreatic beta cells in the rat and guinea pig (Kirchgessner & Liu 1999, Adeghate *et al.* 2010) and in glucagon-producing alpha cells in rat islets (Gonc^z *et al.* 2008). Furthermore, orexin A immunoreactivity was also detected in 65.8% of human beta and 9.8% of alpha cells (Nakabayashi *et al.* 2003). Others confirmed that only orexin A is present in human beta and PP but not in alpha cells and delta cells (Ehrstrom *et al.* 2005). These two studies used different anti-orexin A antibodies. Several studies investigated the impact of diabetes on OXR1 expression in pancreatic islets. Adeghate *et al.* reported an increased number of OXR1-positive cells in pancreatic islets in STZ diabetic and T2DM Goto-Kakizaki rats as compared to Wistar rats (Adeghate & Hameed 2005, Adeghate *et al.* 2010). Noteworthy, healthy animals express OXR1 mostly in insulin-immunoreactive cells, whereas diabetic animals mostly in glucagon-immunoreactive cells. The authors of this study suggest that, at least in type 1 diabetic rats, increased expression of OXR1 in pancreatic islets may be triggered by low intracellular glucose content, which occurs in the majority of cell in the absence of insulin (Adeghate *et al.* 2010). In summary, in pancreatic beta cells OXR1 but not OXR2 is present on protein level. Similar to beta cells, alpha cells also produce OXR1 protein, whereas OXR2 is absent. Therefore, these findings indicate that only OXR1 can be relevant in controlling functions of alpha and beta cells including insulin and glucagon secretion. Nevertheless, it must be considered that protein levels of OXR2 could be present in endocrine cells of pancreatic islets; however, the antibodies are not sensitive enough to identify OXR2 in these cells. Finally, it is worth to notice, that, in addition to alpha and beta cells, pancreatic islets contain many other endocrine cells such as delta, epsilon and PP, which produce somatostatin, ghrelin and pancreatic

polypeptide, respectively (Steiner *et al.* 2010). Expression and distribution of orexin receptors in these cells remain to be investigated.

Regulation of orexin secretion in humans and rodents

The secretion of insulin and glucagon from pancreatic beta and alpha cells is precisely regulated by glucose (MacDonald *et al.* 2005, Walker *et al.* 2011). Ouedraogo *et al.* found that orexin A secretion from isolated rat pancreatic islets increases at low glucose (2.8 mM), while at high concentration of glucose (16.7 mM), orexin A release decreases (Ouedraogo *et al.* 2003). In contrast, there was no difference in orexin A secretion from rat islets at 2.8, 10 and 16.7 mM glucose (Arafat *et al.* 2014), suggesting that orexin A secretion from pancreatic islets is glucose independent. On the other hand, application of glucagon reduces orexin A secretion during static incubation of rat islets at low as well as high glucose concentrations. Reduction of orexin A release by glucagon was not reversed by co-incubation with insulin secretion blocker diazoxide (Arafat *et al.* 2014), suggesting that inhibition of orexin A release by glucagon is rather insulin independent. Nevertheless, co-incubation with anti-insulin antibody inhibits orexin A secretion from rat pancreatic islets, which suggests that insulin stimulates orexin A secretion.

The relevance of glucagon in suppressing orexin A levels was also confirmed *in vivo*. We reported that administration of glucagon reduces circulating levels of orexin A in healthy lean and type 1 diabetic humans as well as in obese non-diabetic mice (Arafat *et al.* 2014). Furthermore, the lack of any correlation between time-dependent changes of glucose and orexin A levels confirms that in the peripheral tissues, glucose is probably not relevant in modulating orexin A secretion from pancreatic islets and its levels in blood circulation. Overall, considering opposite effects of orexin A and glucagon on blood glucose levels (reduction vs rise), glucagon may prevent an excessive decline of blood glucose concentration in response to orexin A. Furthermore, glucagon can stimulate lipolysis (Heckemeyer *et al.* 1983, Perea *et al.* 1995), whereas orexin A suppresses this process (Digby *et al.* 2006, Skrzypski *et al.* 2011). Thus, inhibition of orexin A secretion by glucagon may be relevant event for orexin A-modulated lipid metabolism. Furthermore, it can partially explain the lack of orexin A at promoting lipogenesis *in vivo*.

As compared to orexin A, little is known regarding orexin B expression in pancreatic islets. Orexin B is detectable in pancreatic beta cells in rat islets as well as in nerves in the intrapancreatic blood vessels (Adeghate & Hameed 2011). In contrast to orexin A, nothing is known about the modulation of orexin B production and secretion from pancreatic islets.

The role of orexins in controlling insulin and glucagon secretion

Acute subcutaneous injection as well as prolonged administration of orexin A and orexin B leads to increase in blood insulin levels in rats (Nowak *et al.* 2000, Switonska *et al.* 2002). *In vitro* perfusion study showed that orexin A enhances insulin secretion from rat pancreas (Nowak *et al.* 2000). Stimulation of insulin secretion by orexin A was confirmed by an independent study conducted on perfused rat pancreas by our laboratory (Gonczi *et al.* 2008). In line with these data, Chen *et al.* reported a significant increase in insulin secretion from rat INS-1 insulinoma cells incubated with orexin A (Chen *et al.* 2013). In addition, orexin B is able to stimulate insulin release from pancreatic tissue fragments derived from healthy and STZ diabetic rats (Adeghate & Hameed 2011). Elevation of plasma insulin levels was also reported in humans during orexin A infusion (Ehrstrom *et al.* 2005).

Park *et al.* reported that orexin A stimulates insulin secretion from murine pancreatic islets *in vitro* as well as *in vivo* (Park *et al.* 2015). Adenylate cyclase and ryanodine receptors confer the effects of orexin A on insulin exocytosis (Park *et al.* 2015). In addition, we recently reported that orexin A-stimulated insulin secretion from INS-1E cells can be blocked by nonspecific transient receptor potential channels blockers such as La³⁺ and ruthenium red (Skrzypski *et al.* 2016). Thus, orexin A stimulation of insulin secretion is mediated via cAMP- and calcium-dependent mechanisms.

It must be pointed out that contradictory data regarding the role of orexin in regulating insulin secretion were reported. Tsuneki *et al.* found that exogenous orexin A lowers blood glucose levels in fasted healthy and STZ diabetic mice without affecting insulin concentration (Tsuneki *et al.* 2002). Ehrstrom *et al.* reported in rats that insulin levels were unaffected by exogenous orexin A (Ehrstrom *et al.* 2004). The lack of orexin A effects on insulin secretion during static incubation of rat pancreatic islets was also reported by Colombo *et al.* (2003).

Ouedraogo *et al.* reported that orexin A can reduce insulin secretion from isolated rat islets (Ouedraogo *et al.* 2003).

Regarding the regulation of glucagon secretion by orexin A, more consistent findings were reported. Orexin A suppressed glucagon secretion from *in situ*-perfused rat pancreas, isolated rat pancreatic islets and glucagon-producing hamster InR1-G9 cell line (Gonczi *et al.* 2008). Very recent work demonstrated that orexin A decreases glucagon secretion from porcine pancreatic islets *in vitro* (Sassek *et al.* 2017). On the other hand, others found that orexin A stimulates glucagon release from rat pancreatic islets (Ouedraogo *et al.* 2003). Human study showed no changes in plasma glucagon levels during orexin A infusion (Ehrstrom *et al.* 2005). It is difficult to explain these inconsistencies. Nevertheless, as discussed in the work by Gonczi *et al.*, it cannot be excluded that different experimental settings and methods of glucagon evaluation (ELISA vs RIA) could contribute to these discrepancies (Gonczi *et al.* 2008).

Orexin B enhanced glucagon release from pancreatic tissue fragments derived from non-diabetic rats, without any effects in tissue fragments derived from STZ diabetic rats (Adeghate & Hameed 2011). In InR1-G9 cells, orexin A reduced glucagon mRNA expression via PI3-K/PKB- and FOXO-1-dependent pathways (Gonczi *et al.* 2008).

Taken together, 20 years of studies addressing the role of orexins in controlling insulin secretion provided inconsistent results. Nevertheless, the majority of studies showed that orexin A stimulates insulin exocytosis from beta cells *in vitro* and *in vivo*, in different species including rats, mouse and pigs as well as in insulin-producing cell lines. Furthermore, recent studies addressing mechanism by which orexin A induces insulin release showed that stimulation of calcium and cAMP, two important second messengers involved in insulin exocytosis, is relevant in this context (Prentki & Matschinsky 1987). Thus, these results strongly support the conclusion that orexin A belongs to insulin secretagogues.

The effects of orexin on proliferation and apoptosis of beta cells

We and others reported that orexin A stimulates proliferation of insulin-producing rat INS-1 and INS-1E beta cells (Chen *et al.* 2013, Skrzypski *et al.* 2016). OXR1-activated PI3-K/PKB-dependent mechanism appears to confer the effects of orexin A on beta cell proliferation (Chen *et al.* 2013). Using a pharmacological inhibitor of ERK1/2 signaling, we showed that this pathway is relevant

in mediating the proliferative activity of orexin A in INS-1E cells (Skrzypski *et al.* 2016). Taken together, PI-3K and ERK1/2 pathway are involved in orexin A-induced insulin-producing cell proliferation.

Furthermore, orexin A is able to protect INS-1 cells from apoptotic death induced by serum deprivation (Chen *et al.* 2013). This effect is mediated via OX1 and PKB (Chen *et al.* 2013). Moreover, orexin A can protect INS-1E cells and freshly isolated rat pancreatic islets from TNF- α and palmitic acid-induced apoptosis (Kaczmarek *et al.* 2017). A recent study showed that antiapoptotic effects of orexin A in endocrine cells of pancreas are not restricted to rodents, only. It was shown that, by interacting with porcine isolated pancreatic islets, orexin A stimulates islets viability and protects them from staurosporine-induced apoptosis via ERK1/2-dependent signaling (Sassek *et al.* 2017).

The late stage of T2DM is characterized by the loss of beta cells, increased insulin resistance and inadequate secretion of insulin (Prentki & Nolan 2006). Moreover, the ratio of pancreatic alpha to insulin-producing cells increases in T2DM patients (Henquin & Rahier 2011). Several nongenetic animal models of T2DM are known, one of which can be generated using a single injection of STZ together with prolonged HFD feeding (Luo *et al.* 1998, Reed *et al.* 2000). In this animal model, induction of diabetes by STZ/HFD leads to beta cell loss and hyperglycemia. Alpha cell mass increases in these diabetic animals, reflecting morphological abnormalities of the endocrine pancreas reported in humans. Continuous subcutaneous infusion of orexin A for 4 weeks into rats with STZ/HFD (T2DM) diabetes attenuates the reduction of beta cell area and enhanced insulin mRNA expression control (Kaczmarek *et al.* 2017). In contrast, orexin A reduces alpha cells area and glucagon mRNA expression in T2DM rats. In agreement with the previous studies showing that orexin promotes insulin sensitivity (Funato *et al.* 2009) and increases glucose uptake (Skrzypski *et al.* 2011), we found that orexin A-treated rats displayed attenuated insulin resistance and reduced hyperglycemia (Kaczmarek *et al.* 2017).

Overall, these data suggest that orexin receptor signaling in pancreatic alpha and beta cells may protect from pancreatic islet dysfunctions and beta cell loss in T2DM. This notion is supported by our findings showing that cleaved caspase 3 production and activity in isolated islets are attenuated in orexin A-treated T2DM animals comparing vehicle-treated rats (Kaczmarek *et al.* 2017). Nevertheless, it should be noticed that another study addressing the role of orexin system components in

pancreatic islets showed that mice lacking orexin have reduced number of cleaved caspase-3-positive cells in pancreatic islets (Adeghate *et al.* 2010). The same study showed that induction of type 1 diabetes mellitus in rats is associated with increased number of OX1- as well as cleaved caspase3-positive cells in pancreatic islets (Adeghate *et al.* 2010). Activation of different caspase cascades including caspase 3 plays a prominent role in stimulating beta cells apoptosis (Hui *et al.* 2004). Thus, attenuation of cleaved caspase 3 production in orexin-deficient mice and positive correlation between OX1 and cleaved caspase 3 suggests potential contribution of orexin receptor in beta cell loss. On the other hand, in the light of our data showing that orexin receptor signaling suppresses beta cell death (Kaczmarek *et al.* 2017), it cannot be excluded that upregulation of OX1 in apoptotic cells is a part of cellular self-defense system.

Since both, T1DM and T2DM, are characterized by beta cell loss (Cnop *et al.* 2005) orexin receptor signaling may be a potential therapeutic target relevant for improving beta cell functions.

Concluding remarks

In summary, orexin A promotes fibroblasts/preadipocytes cell proliferation but not the differentiation into mature adipocytes by acting on rat and murine adipocytes. In contrast, orexin B suppresses 3T3-L1 preadipocytes growth. Both orexin isoforms stimulate differentiation and proliferation of porcine preadipocytes, suggesting that species differences exist. In mature rodent and porcine adipocytes, orexin A stimulates glucose uptake, lipid accumulation and adiponectin production. In humans, OX1 and OX2 are expressed in differentiated white adipocytes. However, recent data indicate that OX1 but not OX2 is present in preadipocytes, whereas both receptors are absent in mature adipocytes. Furthermore, orexin A suppresses lipolysis and stimulates PPARG expression in human and rodent adipocytes. Furthermore, orexin A modulates brown adipose tissue formation and functions in rodents, which is relevant in promoting energy expenditure, negative energy balance and protection from obesity. Concluding the role of orexin A in endocrine pancreas, current vast part of data indicate that orexin A stimulates insulin secretion while it inhibits glucagon release. Furthermore, orexin A stimulates insulin-producing INS-1 and INS-1E cell proliferation. These effects are mediated via OX1.

Orexin A can suppress beta cell loss in type 2 diabetic rats. Activation of orexin protects from diet-induced obesity and improves glucose control and beta cells function in animals. In summary, orexin could represent a novel player in regulating the adipo-pancreatic axis. The complex interaction of orexin with the adipo-pancreatic axis could be relevant in the context of obesity and diabetes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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