

Hypooxytocinaemia in obese Zucker rats relates to oxytocin degradation in liver and adipose tissue

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Abstract

The metabolic action of oxytocin has recently been intensively studied to assess the ability of the peptide to regulate energy homeostasis. Despite the obvious weight-reducing effect of oxytocin observed in experimental studies, plasma oxytocin levels were found to be unchanged or even elevated in human obesity. The aim of our study was to evaluate the changes in the oxytocin system in Zucker rats, an animal model closely mirroring morbid obesity in humans. Plasma oxytocin levels were measured in obese Zucker rats and lean controls by enzyme immunoassay after plasma extraction. The expression of oxytocin and oxytocin receptor (OXTR) was assessed at the mRNA and protein levels by quantitative real-time PCR and immunoblotting respectively. Plasma and tissue activity of oxytocinase, the main enzyme involved in oxytocin degradation, were measured by fluorometric assay using an arylamide derivate as the substrate. Obese Zucker rats displayed a marked reduction in plasma oxytocin levels. Elevated liver and adipose tissue oxytocinase activity was noticed in obese Zucker rats. Hypothalamic oxytocin gene expression was not altered by the obese phenotype. OXTR mRNA and protein levels were upregulated in the adipose tissue of obese animals in contrast to the reduced OXTR protein levels in skeletal muscle. Our results show that obesity is associated with reduced plasma oxytocin due to increased peptide degradation by liver and adipose tissue rather than changes in hormone synthesis. This study highlights the importance of the oxytocin system in the pathogenesis of obesity and suggests oxytocinase inhibition as a candidate approach in the therapy of obesity.

Key Words

- ▶ oxytocin
- ▶ obesity
- ▶ insulin resistance
- ▶ oxytocinase
- ▶ oxytocin receptor

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Introduction

The neuropeptide oxytocin is a hormone with a wide range of central and peripheral effects. Besides its well-known role in labour and lactation (Gimpl & Fahrenholz 2001), oxytocin is released in response to various stress stimuli (Jezova *et al.* 1995) and is considered to be a cardiovascular hormone as well (Gutkowska *et al.* 2000, Ondrejckova

et al. 2009). Central oxytocin action includes behavioural effects and an effect on memory (Gimpl & Fahrenholz 2001). Centrally released oxytocin also plays a role in the regulation of food intake, acting as an anorexigenic peptide through continuation of the melanocortin pathway. The ability of oxytocin to reduce food intake is linked to limitation of

meal size and enhancement of the effectiveness of peripheral satiety signals (Leng *et al.* 2008). Recently, we have shown that peripherally administered oxytocin at a low dose is inefficient at inhibiting food intake and stimulates adipogenesis *in vivo* without affecting adipose tissue mass (Eckertova *et al.* 2011). Other studies have revealed the weight-reducing action of oxytocin treatment due to oxytocin-induced anorexia with a positive effect on glucose tolerance and insulin sensitivity (Deblon *et al.* 2011, Maejima *et al.* 2011, Morton *et al.* 2012, Zhang *et al.* 2013).

In vivo physiological levels of circulating oxytocin depend on its synthesis, receptor-mediated internalisation and degradation by oxytocinase. Oxytocinase (cystinyl aminopeptidase, EC 3.4.11.3) is an enzyme that inactivates oxytocin via hydrolysis of the peptide bonds between cysteine and tyrosine (Rogi *et al.* 1996, Keller 2003, Wallis *et al.* 2007). The liver and kidney have been shown to be responsible for the majority of oxytocin degradation (Sjoholm & Ryden 1969, Fjellestad-Paulsen & Lundin 1996). So far, to our knowledge, no data describing the comprehensive regulation of oxytocin levels in obesity, including peptide synthesis, internalisation and degradation, exist.

Nowadays, clarification the metabolic effects of oxytocin is based on genetic studies (Takayanagi *et al.* 2008, Camerino 2009) and pharmacological insights (Deblon *et al.* 2011, Eckertova *et al.* 2011, Maejima *et al.* 2011). As previous studies mapping the changes in plasma oxytocin levels in obesity have reported conflicting results (Stock *et al.* 1989, Schroeder *et al.* 2009, Morton *et al.* 2012), we aimed to determine whether obesity is associated with plasma oxytocin reduction and whether this change (if any) is a result of altered peptide production or degradation.

The Zucker fatty rat represents a well-established model of human obesity and insulin resistance. Obesity in this animal model is a consequence of spontaneous mutation (*fa*) in the gene encoding the leptin receptor resulting in hyperphagia (Phillips *et al.* 1996). Thus, the obese Zucker rats are leptin resistant and related comorbidities associated with obesity also develop. We believe that the results obtained on oxytocin metabolism in Zucker rats provide an integrative insight into the regulation of the oxytocin system in obesity.

Materials and methods

Animals

Male Zucker fatty rats (*fa/fa*) and their lean controls (+/?) aged 9 weeks were purchased from Harlan (Udine, Italy).

Animals were maintained on 12 h light:12 h darkness cycle and allowed *ad libitum* access to water and standard diet. Overnight-fasted rats were killed by decapitation at the age of 33 weeks. Experimental procedures and animal care were carried out according to the regulations of the Jagiellonian University Ethical Committee on Animal Experiments.

Measurement of plasma oxytocin and selected metabolic parameters

Plasma oxytocin levels were measured in duplicate by enzyme immunoassay (EIA) (Phoenix Pharmaceuticals, Burlingame, CA, USA) after plasma peptide extraction on C-18 SEP COLUMN, following the manufacturer's instruction. Precision of the assay declared by the manufacturer is as follows: intra-assay variation <10%; inter-assay variation <15%. Plasma leptin and insulin levels were evaluated by RIA kits (Millipore, Bedford, MA, USA). Lipid profile determination was performed in the Laboratory Diagnostics Unit of The University Hospital in Krakow using commercially available kits (Roche Molecular Diagnostics). In order to determine glucose tolerance, rats were subjected to intraperitoneal glucose tolerance test (IPGTT) after an overnight fast. The rats were administered an i.p. injection of 50% dextrose (w/v) at a dose of 2 g/kg body weight. The blood glucose was measured in the tail vein blood prior to and 30, 60, 90 and 120 min after glucose administration using a glucometer (Accu-Check Active, Roche Diagnostics). Rats were allowed 2 days to recover from IPGTT prior to decapitation followed by plasma and tissue sample collection.

RNA isolation and real-time PCR

Total RNA was isolated from frozen tissues using an RNeasy Universal Plus Mini Kit (Qiagen) followed by RT using a Maxima First Strand cDNA Synthesis Kit (Thermo Fisher, Waltham, MA, USA). Real-time qPCR was performed on an ABI 7900HT thermal cycler (Applied Biosystems, Life Technologies) using Maxima Sybr Green qPCR Master Mix (Thermo Fisher). Primers used for qPCR are shown in Table 1. Because of the high homology of the oxytocin and vasopressin genes, we used the Oxytocin TaqMan Gene Expression Assay (Rn00564446_g1; Applied Biosystems, Life Technologies) to evaluate hypothalamic oxytocin mRNA expression. Gene expression of fat mass and obesity associated (*Fto*) was determined by TaqMan Gene Expression Assay (Rn01538187_m1; Applied Biosystems, Life Technologies). Obtained data were normalised to expression of the housekeeping gene ribosomal protein S29 (*Rps29*)

Table 1 Primer sequences used for qPCR

Gene	Forward	Reverse
<i>Rn18s</i>	5'-GGGAGGTAGTGACGAAAAATAACAAT-3'	5'-TTGCCCTCCAATGGATCCT-3'
<i>Cd68</i>	5'-CCATCCCCACTTGGCTCTCT-3'	5'-TGCGCTGAGAATGTCCAATG-3'
<i>Glut1</i>	5'-TGTGCAGCAGCCTGTGTATG-3'	5'-CCAGCCAGACCAATGAGATG-3'
<i>Il10</i>	5'-CCCTGGGAGAGAAGCTGAAGA-3'	5'-CACTGCCTTGCTTTTATCTCACA-3'
<i>Oxytocinase</i>	5'-GGCACATCAGTGGTTTGGTAATC-3'	5'-TACTCCATGAAAGTGGCAAAGC-3'
<i>Oxtr</i>	5'-TTCTTCTGCTGCTCTGCTCGT-3'	5'-TCATGCTGAAGATGGCTGAGA-3'
<i>Rps29</i>	5'-GCTGAACATGTGCCGACAGT-3'	5'-GGTCGCTTAGTCCAATTAATGAA-3'
<i>Syt4</i>	5'-CGCGTGGAATTCGATGAAAT-3'	5'-TGTTGGACTTAGCGGATCTTCTC-3'
<i>Tnfa</i>	5'-CCACCACGCTCTTCTGTCTAC-3'	5'-ACCACCAGTTGGTTGTCTTTG-3'

(adipose tissue, skeletal muscle, hypothalamus and liver) or *18s rRNA* (*Rn18s*) (kidney), which were not altered by the obese *fa/fa* genotype.

Protein isolation for western blot and enzyme activity assay

We chose epididymal adipose tissue as a typical representative of visceral fat. In comparison with other visceral fat depots, e.g. mesenteric and/or retroperitoneal, epididymal fat possesses the highest adipocyte cell size due to lower proliferative capacity. In addition, triglyceride turnover and oxidative stress predominate in epididymal fat tissue. Its enlargement in obesity contributes more significantly to overall metabolic disturbances (DiGirolamo *et al.* 1998, Sackmann-Sala *et al.* 2012).

Frozen adipose tissue and skeletal muscle samples were homogenised in ice-cold lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40 (v/v), 0.5% sodium deoxycholate (w/v), 0.1% SDS (w/v), 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride, 5 µg/ml leupeptin and 5 µg/ml aprotinin). Homogenates were placed on ice for 2 h with occasional mixing followed by centrifugation at 16 000 *g*/20 min/4 °C. The supernatant was collected and used for western blot analysis. To evaluate the amount of phosphorylated eukaryotic elongation factor 2 (EEF2), adipose tissue samples were homogenised in 25 mM HEPES buffer with a mixture of phosphatase inhibitors (10 mM sodium fluoride, 1 mM sodium orthovanadate and 30 mM sodium pyrophosphate) as described previously (Eckertova *et al.* 2011).

To determine enzymatic activity in the liver, kidney cortex, epididymal adipose tissue and musculus quadriceps membrane fraction, tissues were homogenised by a glass-Teflon homogeniser in a buffer containing 10 mM Tris-HCl, pH 7.4, and 0.25 M sucrose without protease inhibitors. Homogenates were then centrifuged at 1000 *g* for 10 min at 4 °C to remove the nuclear fraction and cell debris.

To obtain the membrane fraction, tissue supernatants underwent centrifugation at 16 000 *g*/15 min/4 °C. Pellet was resuspended in 10 mM Tris, pH 7.4, and solubilised pellets containing the tissue membrane fraction were used for cystinyl aminopeptidase (oxytocinase) activity measurements. The protein concentration in all samples was determined by the BCA method (Sigma Aldrich).

Western blot

Samples were loaded and subjected to SDS-PAGE on 10% polyacrylamide gels. After electrophoretic separation, proteins were electro-transferred to a low-fluorescence PVDF membrane (Millipore). Equal loading was confirmed by Ponceau S staining of blotted membranes. Blots were blocked in 5% non-fat milk (w/v) in Tris-buffered saline for 1 h at room temperature and then incubated overnight at 4 °C with primary antibody against oxytocin receptor (OXTR; H-60; sc-33209; Santa Cruz Biotechnology) diluted 1:400, phosphorylated EEF2 Thr56 (#2331; Cell Signaling Technology, Danvers, MA, USA) diluted 1:1000 and/or total EEF2 (#2332 Cell Signaling Technology) diluted 1:1000 in blocking buffer containing 0.1% Igepal. β-actin (8H10D10; #3700; Cell Signaling Technology) diluted 1:2000 or β-tubulin (926-42211; Li-Cor Biosciences, Lincoln, NE, USA) diluted 1:2000 were used as endogenous loading controls. After membrane washing, the signal of fluorescently labelled secondary anti-rabbit (#5151) and/or anti-mouse (#5257) antibodies (Cell Signaling Technology) diluted 1:15 000 was detected using the Odyssey infrared imaging system (LI-COR Biosciences). Quantification of protein level was performed using Odyssey IR imaging system software version 2.0.

Measurement of cystinyl aminopeptidase activity

Cystinyl aminopeptidase activity was measured fluorometrically using an arylamide derivatate as a substrate, as

Table 2 Selected metabolic parameters and adipose tissue phenotype of obese Zucker rats and their lean controls. Relative epididymal white adipose tissue (eWAT) weight was calculated as a ratio of absolute eWAT weight and the corresponding body weight of the animal. Expression of the gene of interest was normalised to that of the housekeeping gene encoding ribosomal protein S29 (*Rps29*). Data are presented as mean \pm s.e.m. Differences between obese ($n=7$) and lean ($n=6$) Zucker rats were analysed by Student's *t*-test

	Lean (+/?)	Obese (fa/fa)
Body weight (g)	457.3 \pm 8.7	698.7 \pm 16.1 [†]
Plasma leptin (ng/ml)	6.3 \pm 0.7	94.9 \pm 12.9 [†]
Plasma insulin (ng/ml)	1.4 \pm 0.2	14.1 \pm 2.0 [†]
Fasting glycaemia (mmol/l)	6.4 \pm 0.2	6.9 \pm 0.2
2-h glycaemia (mmol/l)	6.3 \pm 0.3	9.5 \pm 1.02 [*]
Triglycerides (mmol/l)	0.78 \pm 0.04	4.47 \pm 0.91 [†]
Cholesterol (mmol/l)	2.53 \pm 0.06	7.06 \pm 0.39 [†]
LDL:HDL ratio	0.29 \pm 0.01	3.44 \pm 0.51 [†]
Relative eWAT weight (%)	1.37 \pm 0.04	2.84 \pm 0.15 [†]
<i>Cd68</i> mRNA (arbitrary units)	0.10 \pm 0.03	0.62 \pm 0.19 [*]
<i>Tnfa</i> mRNA (arbitrary units)	0.55 \pm 0.05	0.98 \pm 0.10 [†]
<i>Il10</i> mRNA (arbitrary units)	0.41 \pm 0.09	0.17 \pm 0.04 [*]
<i>Glut1</i> mRNA (arbitrary units)	0.22 \pm 0.04	0.53 \pm 0.07 [†]

* $P < 0.05$; [†] $P < 0.01$ and [‡] $P < 0.001$. *Glut1*, glucose transporter 1; *Il10*, interleukin 10; *Oxtr*, oxytocin receptor; *Syt4*, synaptotagmin 4; *Tnfa*, tumour necrosis factor alpha.

described previously (Segarra *et al.* 2002). Briefly, plasma samples or the tissue membrane fraction were mixed with substrate solution containing 100 mM arylamide derivate cystinyl- β -naphthylamide (Sigma Aldrich), 10 mg/100 ml both BSA and DTT in 50 mM Tris-HCl, pH 6.0. The 96-well plate containing samples was placed in a fluorimeter (FLUOstar OPTIMA FL, BMG LabTech, Ortenberg, Germany) pre-heated to 37 °C and enzyme kinetics were measured during 1 h as the amount of β -naphthylamine released as a result of enzymatic activity at wavelengths of 412 nm (emission) and 345 nm (excitation). Aminopeptidase activity is expressed as micromoles per liter of cys- β -naphthylamide hydrolysed per minute per milligram of protein.

Statistical analysis

Data are presented as mean \pm s.e.m. Data were tested for normality by the Kolmogorov-Smirnov test. Differences between lean and obese animals were analysed by Student's *t*-test. When data displayed a non-normal distribution, the Mann-Whitney *U* test was used to analyse differences between experimental groups. The Pearson correlation test was used to analyse correlations between variables. The differences among groups were considered to be statistically significant when $P < 0.05$.

Results

Obese Zucker rats displayed marked hyperleptinaemia, dyslipidaemia, hyperinsulinaemia and impaired glucose tolerance assessed by 2-h glycaemia during IPGTT but normal fasting glycaemia. In adipose tissue, upregulation of *Cd68* mRNA, a marker of macrophages, elevated mRNA levels of pro-inflammatory cytokine tumour necrosis factor alpha (*Tnfa*) and at the same time decreased expression of anti-inflammatory cytokine interleukin 10 (*Il10*) were detected. Obesity in Zucker fatty rats was associated with increased mRNA expression of glucose transporter 1 (*Slc2a1* (*Glut1*)) in adipose tissue. Baseline characteristics of experimental groups are shown in Table 2.

We observed a 40% reduction of plasma oxytocin levels (Fig. 1) in obese Zucker rats when compared with their lean controls. No correlation between plasma oxytocin and body weight within each group was found.

In order to evaluate oxytocin degradation and bioavailability, we measured the gene expression and activity of oxytocinase (cystinyl aminopeptidase) in plasma and peripheral tissues. Interestingly, the obese phenotype resulted in increased oxytocinase mRNA expression and activity in liver (Fig. 2A and B). In the kidney cortex, we observed increased oxytocinase expression at the mRNA level (Fig. 2A), but this change did not lead to elevated enzyme activity in the membrane fraction (Fig. 2B). Despite the unchanged oxytocinase mRNA expression in both adipose tissue and skeletal

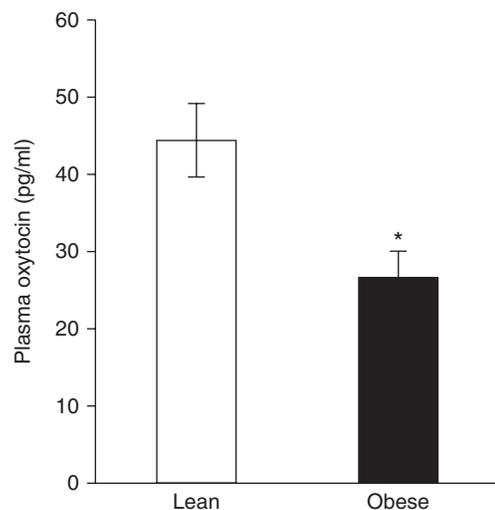
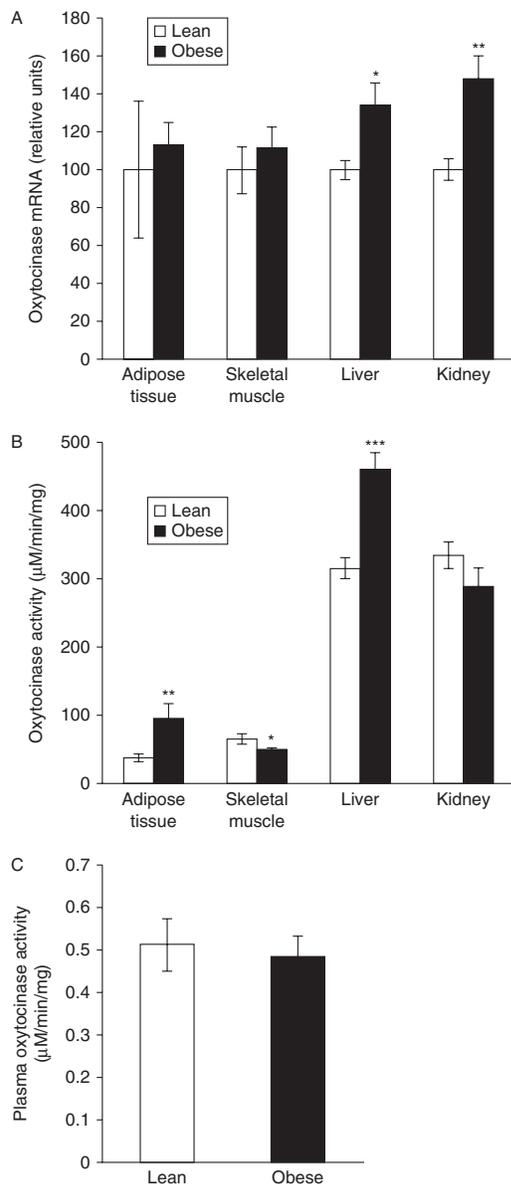


Figure 1

Plasma oxytocin levels. Oxytocin levels in plasma of lean and obese Zucker rats evaluated by EIA after peptide extraction. Data are expressed as mean \pm s.e.m. Differences between obese ($n=7$) and lean ($n=6$) Zucker rats were analysed by Student's *t*-test. * $P < 0.05$.

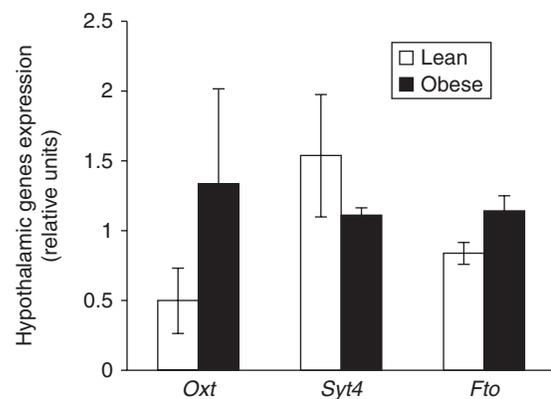
**Figure 2**

Oxytocinase expression and activity in peripheral tissues and plasma. (A) Oxytocinase mRNA expression levels in epididymal adipose tissue, musculus quadriceps, liver and kidney cortex were determined by real-time PCR. Data were normalised to those for the housekeeping genes encoding ribosomal protein S29 (adipose tissue, skeletal muscle and liver) and *Rn18s* (kidney cortex) whose expression was not significantly changed by the obese phenotype. Results are presented as a percentage change against control within the tissue and expressed as mean \pm s.e.m. Data were analysed by Student's *t*-test. * $P < 0.05$ and ** $P < 0.01$. (B) Oxytocinase activity in the plasma membrane fractions of adipose tissue, skeletal muscle, liver and kidney cortex. Data are expressed as mean \pm s.e.m. Differences between obese ($n = 7$) and lean ($n = 6$) Zucker rats were analysed by Student's *t*-test or Mann–Whitney *U* test, when appropriate. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. (C) Plasma oxytocinase activity in lean and obese Zucker rats determined by fluorometric assay using an arylamide derivative as a substrate. Data are expressed as mean \pm s.e.m. Differences between obese ($n = 7$) and lean ($n = 6$) Zucker rats were analysed by Student's *t*-test.

muscle (Fig. 2A), we observed a decline in skeletal muscle and an increase in adipose tissue oxytocinase activity in the plasma membrane fraction of obese Zucker rats (Fig. 2B) respectively. No difference in plasma oxytocinase activity between obese Zucker rats and lean controls was observed (Fig. 2C).

With the purpose of assessing oxytocin production, we measured hypothalamic oxytocin gene expression in lean and obese Zucker rats (Fig. 3). We observed more than twofold increase in oxytocin expression in the obese animals, although this difference did not reach significance due to high variability within the groups ($P = 0.534$). Gene expression of synaptotagmin 4 (*Syt4*), a negative regulator of oxytocin exocytosis, was not altered by the obese phenotype (Fig. 3). Hypothalamic expression of fat mass and obesity associated (*Fto*), a proposed transcription co-factor involved in regulation of oxytocin expression, displayed a tendency to increase ($P = 0.064$) in animals with the obese phenotype (Fig. 3).

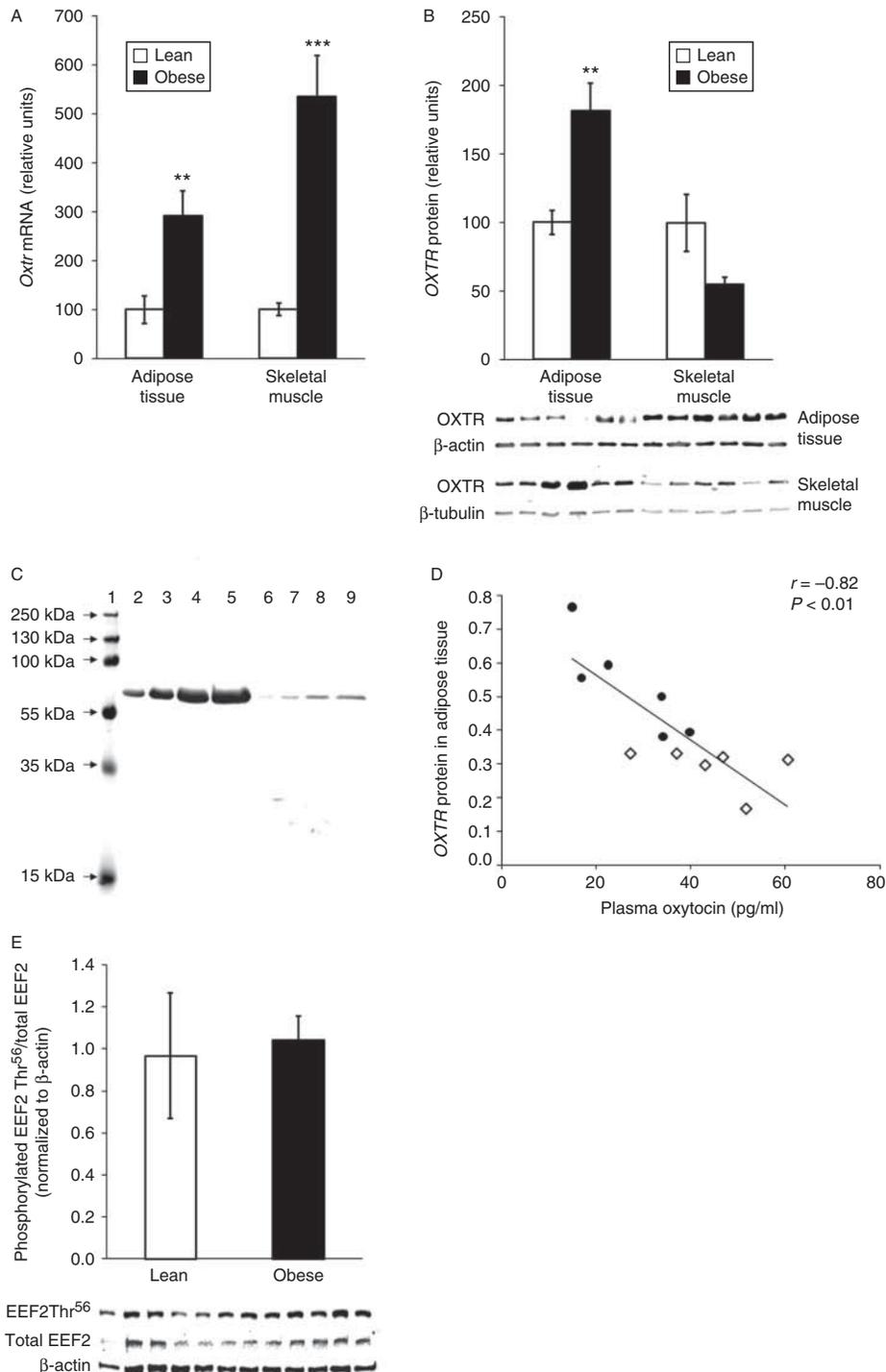
We detected *Oxtr* mRNA as well as protein in epididymal adipose tissue and musculus quadriceps of rats (Fig. 4). In epididymal adipose tissue, both *Oxtr* mRNA (Fig. 4A) and protein level (Fig. 4B) were significantly upregulated in obese Zucker rats. However, despite the sevenfold increase in *Oxtr* mRNA level (Fig. 4A), we observed a more than 40% reduction in OXTR protein level (Fig. 4B) in the skeletal muscle of obese rats, although this change did not reach statistical significance ($P = 0.066$). In order to compare OXTR expression in adipose tissue and skeletal muscle, we loaded different

**Figure 3**

Expression of selected hypothalamic genes. Expression of genes of interest in the hypothalamus of lean and obese Zucker rats measured by real-time PCR. Data were normalised to that for the housekeeping gene encoding ribosomal protein S29 whose expression was not significantly changed by the obese phenotype. Data are expressed as mean \pm s.e.m. Differences between obese ($n = 7$) and lean ($n = 6$) Zucker rats were analysed by Student's *t*-test.

quantities of adipose tissue and skeletal muscle homogenates from the same lean individual for western blot analysis. We found that OXTR protein expression was approximately ninefold higher (9.21 ± 0.91) in adipose tissue than in skeletal muscle (Fig. 4C). When correlating OXTR protein with oxytocin plasma levels, a highly

significant negative relation was found in epididymal adipose tissue (Fig. 4D) in contrast to no correlation in quadriceps muscle (data not shown). Moreover, a strong positive correlation ($r=0.83$; $n=13$; $P<0.001$) was found between *Oxtr* and *Cd68* mRNA expression in adipose tissue. This correlation disappeared when the lean group



alone was analysed ($r = -0.30$; $n = 6$; $P = 0.56$) but persisted within the obese group ($r = 0.82$; $n = 7$; $P < 0.05$). To consider entire epididymal adipose tissue sensitivity to plasma oxytocin, we determined phosphorylation of the oxytocin downstream signalling molecule, EEF2. Western blot analysis revealed no changes in phosphorylation of EEF2 in the obese phenotype animals (Fig. 4E).

Discussion

This study reveals a modification of the oxytocin system without clear implications in the oxytocin signalling in 33-week-old obese insulin-resistant Zucker rats. In spite of being a genetic model of obesity (Phillips *et al.* 1996), Zucker fatty rats represent an appropriate model for studying human obesity, as related metabolic comorbidities associated with obesity also develop. In addition, the adipose tissue of obese Zucker rats displays characteristics typical of human obesity such as tissue inflammation and hypoxia.

The obesity-associated hypooxytocinaemia observed in our study is consistent with the previously observed tendency towards a reduction of plasma oxytocin levels in high-fat-diet-induced obesity (Morton *et al.* 2012).

Recent research has linked circadian arrhythmicity to obesity and related diseases (Bray & Young 2007, Froy 2010). In rodents, a high-fat diet promotes daytime rather than night-time caloric intake leading to feeding circadian arrhythmicity. It has been well documented that hyperphagia in obese Zucker rats is manifested by increasing meal size during the light phase (Becker & Grinker 1977, Fukagawa *et al.* 1992, Mistlberger *et al.* 1998). Hypothalamic release of anorexigenic oxytocin displays a

diurnal rhythm of daytime rise and night-time decline, which is abrogated in high-fat-diet-induced obesity (Zhang & Cai 2011). As we measured oxytocin in blood obtained from decapitation at daytime, the reduced oxytocinaemia corresponds to the observed daytime decline in obese animals and supports that attenuated oxytocin manifestation contributes to energy imbalance.

Obese and normal-weight men display similar basal oxytocin levels and weight reduction is not accompanied by a change in plasma oxytocin (Coiro *et al.* 1988). On the other hand, Stock *et al.* (1989) showed that the plasma oxytocin levels were fourfold higher in obese subjects compared with controls regardless of sex. Following gastric banding and substantial weight loss of the obese individuals, the oxytocin levels in plasma were reduced.

The absence of a correlation between plasma oxytocin and body weight within a single group observed in this study is in line with a previous result showing no relation between oxytocinaemia and BMI or body fat (Hoybye *et al.* 2003). This observation implies that obesity affects plasma oxytocin secondarily rather than changes in oxytocin levels affecting body weight. On the other hand, studies using oxytocin and/or OXTR-deficient mice have shown that a lack of oxytocin action is accompanied by the development of obesity and impaired glucose tolerance (Takayanagi *et al.* 2008, Camerino 2009). In addition, the beneficial effects of oxytocin treatment, such as weight loss, have been well documented recently (Deblon *et al.* 2011, Maejima *et al.* 2011). These facts imply at least a partial role of oxytocin in the development of obesity.

Recently, Szeto *et al.* (2011) emphasised a critical need to establish valid and reliable methods to measure plasma oxytocin. In this regard, plasma extraction is the basic

Figure 4

Expression of oxytocin receptor (OXTR) in adipose tissue and skeletal muscle. (A) *Oxtr* mRNA expression in epididymal adipose tissue and musculus quadriceps of lean and obese Zucker rats determined by real-time PCR. Data were normalised to those for the housekeeping gene encoding ribosomal protein S29 whose expression was not significantly changed by the obese phenotype neither in adipose tissue nor in skeletal muscle. Results are presented as a percentage change against control levels within the tissue and expressed as mean \pm s.e.m. Differences between obese ($n = 7$) and lean ($n = 6$) Zucker rats were analysed by Student's *t*-test or Mann-Whitney *U* test, when appropriate. $**P < 0.01$ and $***P < 0.001$. (B) Protein level of OXTR in total homogenates of epididymal adipose tissue and musculus quadriceps evaluated by western blot. Densitometric quantification of results for signals corresponding to OXTR were normalised to those for the endogenous loading controls β -actin (adipose tissue) or β -tubulin (skeletal muscle). Representative blots are shown at the bottom of the figure. From the left, the first six bands represent lean and the next six ones obese animals. Results are presented as a percentage change against control levels within the tissue and expressed as mean \pm s.e.m. Differences between obese ($n = 6$) and lean

($n = 6$) Zucker rats were analysed by Student's *t*-test. $**P < 0.01$.

(C) A comparison of OXTR protein expression in adipose tissue and skeletal muscle of the same lean individual evaluated by western blot. Lane 1: protein ladder; lanes 2–5: epididymal adipose tissue total homogenate (5, 10, 20 and 30 μ g); lanes 6–9: quadriceps muscle total homogenate (5, 10, 20 and 30 μ g). The expected molecular weight of OXTR protein was 66 kDa (antibody datasheet sc-33209). (D) Correlation between plasma oxytocin levels and OXTR protein in epididymal adipose tissue of lean (open squares) and obese rats (filled circles). Data were statistically analysed using the Pearson correlation test ($n = 12$). (E) Protein level of eukaryotic elongation factor 2 (EEF2) in epididymal adipose tissue of lean and obese Zucker rats evaluated by western blot. The phosphorylated to total EEF2 ratio was normalised to the endogenous loading control β -actin. Representative blots are shown at the bottom of the figure. From the left, the first six bands represent lean and the next six ones obese animals. Results are expressed as mean \pm s.e.m. Differences between obese ($n = 6$) and lean ($n = 6$) Zucker rats were analysed by Student's *t*-test.

condition in order to remove higher molecular weight immunoreactive contaminants from the sample before an antibody-based oxytocin assay is employed (Szeto *et al.* 2011). However, even extracted plasma samples might contain low-molecular-mass immunoreactive contaminants, most probably the degradation products of oxytocin. Mass-spectrometry-based methods seem to be a good, yet expensive and not always accessible, solution for reliable oxytocin quantification. In summary, methodological differences in oxytocin determination might be responsible for the variability in oxytocin response to obesity described in different studies. Interpretation of oxytocin data must therefore take into account the limitations of the quantification methods employed. In this study, plasma samples pre-purified on a C-18 column were used for EIA evaluation.

Oxytocinase regulates circulating oxytocin levels and its biological activity in tissues via inactivation of the peptide by hydrolysis of cysteine-tyrosine bonds (Rogi *et al.* 1996, Keller 2003). Importantly, increased oxytocinase expression and activity in the liver of obese Zucker rats implies a higher rate of oxytocin cleavage, indicating limited bioavailability of the peptide due to its degradation. In contrast, in the kidney, despite the elevated levels of oxytocinase mRNA, no changes in the enzyme activity were observed. It seems that the kidney is not involved in the elevated oxytocin degradation in obesity. Similarly, plasma oxytocinase activity did not participate in increased oxytocin degradation in our model of obesity. The same results were found by Ramirez *et al.* (1998) in olive-oil-fed mice.

Oxytocinase has been shown to be identical to insulin-regulated aminopeptidase (IRAP). In insulin-responsive tissues, IRAP is almost exclusively co-localised with the insulin-regulated glucose transporter GLUT4, which is retained in intracellular compartments in the basal state or translocates with GLUT4 to the plasma membrane upon insulin stimulation (Keller *et al.* 1995, Rogi *et al.* 1996). The decline in IRAP/oxytocinase activity in the skeletal muscle membrane fraction of obese rats confirms impaired muscle insulin sensitivity in these rats. The increased IRAP/oxytocinase activity in the epididymal adipose tissue membrane fraction of obese rats is consistent with previously described abnormal subcellular adipocyte IRAP/oxytocinase distribution and accumulation in the plasma membrane fraction under basal conditions when glucose homeostasis is disrupted in humans (Maianu *et al.* 2001). Concomitantly with the appearance of IRAP/oxytocinase at the cell surface, aminopeptidase activity toward extracellular substrates increases (Keller 2003). Thus, the increased oxytocinase

activity in the plasma membrane fraction implies higher oxytocin processing in the epididymal adipose tissue of obese rats. Considering the multiple fat mass expansions in obesity, increased oxytocinase activity in the adipose tissue of obese Zucker rats may significantly contribute to the low plasma levels of oxytocin.

Our hypothesis that oxytocin degradation rather than lowered oxytocin production and/or secretion accounts for decreased plasma oxytocin levels in obesity is supported by the unchanged expression of the oxytocin gene as well as *Syt4* in the hypothalamus of obese Zucker rats compared with lean ones. Recently, it has been clearly demonstrated that *Syt4* is predominantly expressed in oxytocin neurons and serves as a negative regulator of oxytocin release (Zhang *et al.* 2011). In contrast, Zhang *et al.* (2011) observed upregulation of both *Syt4* and the oxytocin gene together with decreased oxytocin release in hypothalami of obese mice fed with a high-fat diet. However, oxytocin degradation was not studied in the above-mentioned paper. Thus, one apparently cannot exclude the contribution of peripheral oxytocin degradation to the decline in plasma oxytocin in the mouse model of obesity induced by a high-fat diet. The product of the fat mass and obesity associated gene has been shown to colocalise with oxytocin neurons. Moreover, FTO positively regulates expression of the gene encoding oxytocin (Olszewski *et al.* 2011). Thus, the hypothalamic *Fto* mRNA level is consistent with observed the unchanged oxytocin expression. We might conclude that hypothalamic oxytocin production and release is not affected in obese Zucker rats.

The physiological effects of oxytocin are mediated via interaction with its G_q protein-coupled receptor. Only one isoform of the OXTR, widely expressed throughout the whole body, has been identified (Devost *et al.* 2008). The OXTR was also detected in 3T3-L1 pre-adipocytes/adipocytes and isolated rat fat cells (Bonne & Cohen 1975, Boland & Goren 1987, Schaffler *et al.* 2005), as well as in the C2C12 mouse myoblast cell line and human myoblasts (Breton *et al.* 2002, Lee *et al.* 2008); however, OXTR was not identified in the skeletal muscle of rams (Whittington *et al.* 2001). We confirmed the presence of OXTR *in vivo* in insulin-sensitive tissues at both the mRNA and protein level and found that the receptor expression in these tissues was differentially regulated by obesity. To our knowledge, there are no other studies on the regulation of OXTR expression in adipose tissue and/or skeletal muscle in obesity and insulin resistance. Moreover, this is the first evidence, to our knowledge, that OXTR is much more expressed in adipose tissue than in skeletal muscle. Low expression of OXTR in skeletal muscle could be an

explanation for OXTR not being detected in skeletal muscle of other species (Whittington *et al.* 2001). Based on the marked difference in tissue OXTR expression, we suggest that there is a more pronounced oxytocin effect on adipose tissue than that on skeletal muscle.

Oxytocin stimulates glucose uptake in skeletal muscle cells (Lee *et al.* 2008) and stimulates myoblast fusion and myotube formation (Breton *et al.* 2002). Thus, the decreased plasma oxytocin together with reduced OXTR in the skeletal muscle of Zucker fatty rats may contribute to reduced muscle mass (Kemp *et al.* 2009) and the reduced muscle glucose disposal observed in obesity and insulin resistance (King *et al.* 1992). Based on muscle OXTR protein data and muscle membrane oxytocinase activity, we conclude that there is reduced oxytocin processing in the tissue under the conditions of obesity.

Oxtr mRNA is upregulated during differentiation of 3T3-L1 pre-adipocyte into mature adipocytes (Schaffler *et al.* 2005). Adipose tissue of obese Zucker rats expands both by hypertrophy and by hyperplasia (Johnson *et al.* 1978, Kaplan *et al.* 1980). Therefore, the rise in OXTR levels could be linked to increased adipocyte cell numbers. However, upregulation of the OXTR may simply be an adaptation of adipose tissue to decreased hormone levels in the circulation. This is in accordance with the observed strong negative correlation between plasma oxytocin levels and OXTR protein in epididymal adipose tissue. To assess the sensitivity of adipose tissue to plasma oxytocin, we checked possible oxytocin downstream signalling. Recently, we have clearly demonstrated EEf2 dephosphorylation in rat epididymal adipose tissue upon oxytocin treatment (Eckertova *et al.* 2011). The absence of a change in EEf2 phosphorylation in adipose tissue homogenate of obese Zucker rats indicates similar activation of the oxytocin signalling pathway. We may suppose that decreased plasma oxytocin levels in Zucker fatty rats are compensated for by higher OXTR expression in adipose tissue. However, we cannot exclude the possibility of macrophage infiltration as revealed by upregulated transcription of *Cd68* in adipose tissue of obese rats, which may account for increased OXTR expression. This is supported by the presence of OXTR in macrophages (Szeto *et al.* 2008) and by the observed strong positive correlation between *Cd68* and *Oxtr* in adipose tissue in our study.

In conclusion, our study provides clear evidence that the oxytocin system is significantly altered in 33-week-old Zucker fatty rats, an animal model of obesity and insulin resistance. Reduced plasma oxytocin levels are most probably a result of higher oxytocin degradation by the liver and expanded adipose tissue in obese animals.

In addition, these changes are accompanied by altered expression of the OXTR in adipose tissue and skeletal muscle. Thus, our understanding of the physiological effects of oxytocin is changing as the biological significance of the oxytocin system extends far beyond the reproductive system. The results presented are likely to motivate further research into human obesity as there is a gap in the knowledge regarding the regulation of oxytocinase and OXTR.

Declaration of interest

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

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Author contribution statement

L G prepared major parts of the manuscript and carried out analyses of western blots. L G and K K conducted real-time PCR analyses and data interpretation. A B S and L G carried out measurements of enzyme activity and the literature search. A S carried out analyses of the hypothalamus. M S and L G conducted glucose tolerance tests and biochemical analyses. R O and S Z planned and organised the study and contributed to the revisions and the final drafts of the manuscript. All authors have read and approved the final manuscript.

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