Peripheral insulin administration enhances the electrical activity of oxytocin and vasopressin neurones in vivo

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Abstract

Oxytocin neurones are involved in the regulation of energy balance through diverse central and peripheral actions, and in rats are potently activated by gavage of sweet substances. Here we tested the hypothesis that this activation is mediated by the central actions of insulin. We show that, in urethane-anesthetised rats, oxytocin cells in the supraoptic nucleus show prolonged activation after i.v. injections of insulin, and that this response is greater in fasted rats than in non-fasted rats. Vasopressin cells were also activated, but less consistently. We also show that this activation of oxytocin cells is independent of changes in plasma glucose concentration, and is completely blocked by central (i.c.v.) administration of an insulin receptor antagonist. Finally we replicated the previously published finding that oxytocin cells are activated by gavage of sweetened condensed milk, and show that this response too is completely blocked by central administration of an insulin receptor antagonist. We conclude that the response of oxytocin cells to gavage of sweetened condensed milk is mediated by the central actions of insulin.

INTRODUCTION

Insulin is widely known for its role in glucose homeostasis on peripheral tissues, but its central effects are not yet fully elucidated. Once secreted into the circulation, insulin is transported into the brain by a saturable transport mechanism (1, 2). Both exogenous insulin administration and glucose-stimulated insulin secretion result in a progressive increase of insulin in the CSF in several species, including humans (3-6).Accordingly, insulin concentrations in the cerebrospinal fluid (CSF) correlate with levels in plasma, but they are approximately 15-fold lower than plasma concentrations in fasted rats (3).

In the brain, regions sensitive to insulin include the hypothalamus (7, 8) which contains insulin-responsive neurones in several nuclei (9-11). Amongst these, the insulin receptor (InsR) is abundantly expressed in the supraoptic nucleus (SON) (12-14) which exclusively contains magnocellular oxytocin and vasopressin cells, and intraperitoneal administration of insulin induces the expression of Fos protein in parvo- and magnocellular oxytocin cells of the paraventricular nucleus in rats (15). Explants of the hypothalamo-neurohypophysial system, including the SON and its projections to the posterior pituitary, release oxytocin and vasopressin in response to direct application of insulin (16), and central administration of insulin increases peripheral secretion of oxytocin in mice, by a direct action on oxytocin cells (17).

In addition to their classical roles in reproduction (18, 19), stress (20) and water balance (21), oxytocin and vasopressin have roles in energy homeostasis(22). Both central and peripheral oxytocin administration exert anorexigenic effects, increase energy expenditure, and induce lipolysis (23-26). Peripheral administration of both oxytocin and vasopressin can induce the release of insulin from the pancreas (27-30), and systemically administered oxytocin in humans (administered intranasally) has been reported to curb the meal-related increase in plasma glucose (31), and to improve β-cell responsivity and glucose tolerance in healthy men (32). Studies using well-validated radioimmunoassays in extracted plasma samples (33) indicate that patients with metabolic syndrome exhibit higher circulating oxytocin concentrations than normal individuals (34), and patients with diabetes have higher concentrations of vasopressin, and of copeptin (which is co-secreted with vasopressin) (35, 36).

In this study, we examine whether peripheral (i.v.) administration of insulin affects the electrical activity of oxytocin and vasopressin cells in the SON of urethane-anaesthetised rats. The effect of different feeding states, and consequently different blood glucose concentrations on these responses was also investigated. We investigated the role of brain

InsR in these responses by blocking these receptors using an InsR antagonist. Finally, we tested whether the previously reported enhanced electrical activity of oxytocin cells in response to sweet food gavage (37) was mediated by endogenous insulin release acting on brain InsRs.

METHODS

Animals

We used adult male Sprague–Dawley rats weighing 300-350g. The rats had *ad libitum* access to food and water and were maintained under a 12:12h light/dark cycle (lights on 07.00h) at a room temperature of 20-21°C. In most experiments we used fasted rats to reduce the variability of blood glucose and gastric signals (induced by prior food consumption) which could affect neural activity, so in these experiments the food was removed overnight (~15 h). All procedures were conducted on rats under deep terminal anaesthesia in accordance with the UK Home Office Animals Scientific Procedures Act 1986, and a project licence approved by the Ethical Committee of the University of Edinburgh.

Drugs

Human recombinant insulin solution (cat no. I9278; Sigma-Aldrich Company Ltd., Dorset, UK) was diluted in 0.9% saline (B. Braun, Melsungen, Germany) at 0.25 U/100 µl. Glucose solution was prepared by dissolving 5% glucose (cat no. G5767; Sigma-Aldrich Company Ltd., Dorset, UK) in sterile distilled water. The InsR antagonist S961 (cat no. 051-86; Phoenix Europe GmbH, Karlsruhe, Germany) was dissolved at 0.33 nmol/µl in artificial CSF (NaCl 138 mM, KCl 3.36 mM, $NaHCO_3$ 9.52 mM, Na_2HPO_4 $2H_2O$ 0.49 mM, urea 2.16 mM, $CaCl_2$ 1.26 mM, $MgCl_2$ $6H_2O$ 1.18 mM; pH 7.5).

The responses of SON neurones to systemic insulin were tested by giving an i.v. bolus of 0.75 U/kg. To determine the effect of restoring circulating glucose content in insulin-responsive neurones, 400µl of 5% glucose solution was given i.v. at 200 µl/min. Glucose concentrations were then checked 5 and 20 min later and, if lower than basal values, were corrected by infusing an additional 300 µl and 100 µl of 5% glucose solution, respectively. To investigate the role of central InsRs on insulin responses, the InsR antagonist S961 (38) was given into the third ventricle using a 31-gauge needle (attached behind the bipolar stimulating electrode) inserted through the median eminence; 1 nmole (4.8 µg) of S961 was injected at 1 µl/min. We chose a dose expected to be sufficient to block insulin receptors throughout the brain when given icv, but lower than that needed to antagonise insulin actions if given peripherally. The affinity of S961 for both isoforms of the insulin receptor is close to

that of insulin itself (38). Previous studies have reported that bilateral injections of 100 ng S961 into the arcuate nucleus block the effects of insulin microinjected into the arcuate nucleus on lumbar sympathetic nerve activity in late pregnant rats (39). Studies using the closely related antagonist S661, which has properties indistinguishable from those of S961, indicated that peripheral doses of 30 nmole/kg or more are needed to block the effects of i.v. administration of 30 mmole/kg insulin on blood glucose levels (38). As detailed below, the icv application of S961 in our hands had no significant effect on plasma glucose concentrations.

Sweet condensed milk (SCM) gavage

In fasted rats, a gavage tube was inserted orally into the stomach to deliver a total volume of 5 ml of SCM (Nestle, UK) diluted 50 % v/v in distilled water (40.8 kJ, 1.68g sugar, 0.24g fat) at 0.16 ml/min.

In vivo electrophysiology

Rats were briefly anaesthetised with isoflurane inhalation anaesthesia, and then urethane (ethyl carbamate 25% solution) was injected intraperitoneally (i.p.) at 1.25 g/kg. A femoral vein was cannulated for drug administration and an endotracheal tube was inserted to maintain the airway open, and the SON and the pituitary stalk were exposed by transpharyngeal surgery (40). A bipolar stimulating electrode (SNEX-200X; Clark Electromedical Instruments, Reading, UK) was placed on the pituitary stalk, and a glass microelectrode (~1 μm tip; filled with 0.9% NaCl) was lowered into the SON under visual control for extracellular recording. The signal was amplified using an Axonpatch 200B (Molecular Devices, Sunnyvale, CA, USA) connected to a HumBug 50 Hz noise eliminator (Quest Scientific Instruments Inc., North Vancouver, BC, Canada), and was digitalized with a CED-1401 laboratory interface (Cambridge Electronic Design, Cambridge, UK) connected to a PC running Spike2 software (version 7.20; Cambridge Electronic Design). Most recordings were made from single neurones; in some experiments the spike activity of two cells was recorded simultaneously; in these cases the spikes were discriminated and analysed offline using the waveform function of Spike2 software. Recordings were made between 12:00 and 17:00 h (lights on 07.00-19.00h). Rats were tested only once with insulin.

SON neurones were antidromically identified through stimulation of the pituitary stalk by matched biphasic pulses (1 ms, <1 mA peak to peak), which produce an antidromic spike at a constant latency (~10 ms; Fig.1A). Oxytocin cells were discriminated from continuous-firing vasopressin cells (Fig.1B) by the shape of the interspike interval (ISI) distribution (Fig.1C,D) and by their opposite response to i.v. cholecystokinin (CCK; CCK-8

sulphated cat no. H-2080; Bachem AG, Bubendorf, Switzerland) given at 20 µg/kg, i.e. a transient excitation of oxytocin cells, and no effect or short inhibition of vasopressin cells (Fig.1E, F)(40, 41). CCK was given at the end of the experiments to identify continuously-firing cells.

Recording and blood sampling protocols

Effect of i.v. insulin.

The spontaneous spiking activity of SON neurones was recorded for 20 min (basal activity) and for at least 60 min after i.v. insulin. Blood samples (50 µl) were taken to measure glucose immediately before administration of insulin or vehicle, and 15, 30, 60, 90, and 120 min later.

Effect of restoring circulating glucose content in insulin-responsive neurones.

The basal activity of SON neurones was recorded for 20 min, and for another 30 min after i.v. insulin. Then, glucose was given i.v. and the spike activity recorded for further 30min. Blood glucose concentrations were measured before insulin, 30 min later (i.e. before i.v. glucose), and 5 and 20 min after the first glucose injection. Only rats exhibiting in the last sample a blood glucose concentration within 15% of the value in the basal sample were used.

Blockade of central InsRs

The basal spike activity of SON neurones in fasted rats was recorded for 20 min. Then, S961 was given i.c.v. and spike activity recorded for 15 min. After this, insulin was given i.v. and the spike activity recorded for another 30 min. Blood glucose concentrations were measured using an Accu-Chek Aviva meter (Roche Diagnostics GmbH, Germany) immediately before S961 injection, 15 min later (i.e. before i.v. insulin), and 30 min after i.v. insulin.

Effect of central InsR blockade on SCM-stimulated activity of oxytocin cells

The basal spike activity of SON neurones was recorded for 20 min. Then, rats were injected i.c.v. with either vehicle or S961 and activity recorded for 10 min. After this, SCM was gavaged (over 30 min) and spike activity recorded for 1 h. Blood samples (300 µl) were taken immediately before the i.c.v. injection, 10 min later (i.e. before SCM gavage), and at 30 and 60 min after the start of gavage. Blood glucose concentrations were measured immediately after sampling; then samples were centrifuged in EDTA-coated tubes, and plasma collected and stored at -80°C for insulin measurements using a rat/mouse insulin ELISA kit (cat. EZRMI-13K; EMD Millipore, Billerica, MA, USA).

Hazard Functions

Hazard function displays how the excitability of a neurone changes with the time subsequent to the last spike, indicating the probability of a neurone firing a spike in a given period. For responsive neurones in fasted rats, we constructed ISI histograms in 10-ms bins of the 20-min basal period and the last 30-min after insulin administration. From these, hazard functions (in 10-ms bins) were constructed using the formula: [hazard in bin (t, t + 10)] = [number of ISIs in bin (t, t + 10)]/(number of ISIs of length >t) as described by Sabatier *et al.* (42). Hazard functions plot the incidence of spikes as a proportion of the size of the residual tail of the ISI distribution. When plotted this way, a negative exponential distribution (the distribution characteristic of random events) becomes a constant 'hazard' proportional to the average firing rate. Deviation from this then becomes interpretable as periods of decreased or increased excitability. Consensus hazard functions were calculated from the means of hazard functions.

Statistical analysis.

Data were analysed using Graph Pad Prism 6 (GraphPad Software, CA, USA). Responses to insulin were analysed by comparing the mean firing rate in the 60-min after insulin with the (basal) firing rate over the 20-min control period. The changes were compared using a two-tailed Wilcoxon signed-rank test. The activity of phasic cells was analysed in Spike2; detection of a burst of activity was defined by spike activity lasting at least 5 s and containing >20 spikes followed by >5 s of spike silence between bursts. The mean burst duration, interburst interval and activity quotient (percentage of active time over the total time) over the 20-min basal and 60 min after insulin were compared using Wilcoxon matched-pairs signed-rank test.

The effect of glucose on insulin-responsive cells was analysed by comparing the mean change in firing rate (spikes/s in 10-min bins) before and after glucose (i.e. 0–30 min vs 30–60 min) using Wilcoxon matched-pairs signed-rank test.

The effect of blockade of central InsRs was analysed by testing whether the mean change in firing rate in the 15-min after S961 injection was significantly different from 0 (i.e. from the basal rate), using a two-tailed Wilcoxon signed-rank test. Then the mean change in firing rate over 30-min after insulin was compared with the firing rate in the 15 min after S961 using a two-tailed Wilcoxon signed-rank test. One-way ANOVA followed by *post hoc* Bonferroni test was used to compare glucose profiles.

The mean change in firing rate over 60-min, and glucose profiles between fasted and non-fasted rats were compared using two-tailed Mann Whitney test and two-way ANOVA followed by *post hoc* Bonferroni multiple comparison tests, respectively. We also compared

the change in firing rate to determine whether different treatments affect the responses of SON neurones to insulin, using two-way ANOVA followed by *post hoc* Bonferroni test.

The effect of prior blockade of central InsRs on SCM-induced activity was analysed using a two-tailed Mann Whitney test comparing the mean change in firing rate over 60 min between i.c.v. control- and S961-treated rats. The change in firing rate (in 10-min bins), blood glucose concentrations, and plasma insulin content between the two groups were compared using two-way ANOVA, followed by *post hoc* Bonferroni test.

All data are presented as means ± S.E.M., and statistical significance was set at $P<0.05$ unless otherwise stated.

RESULTS

Oxytocin cells

In both fasted and non-fasted rats, i.v. injections of insulin induced a prolonged increase in the firing rate of oxytocin cells, reaching a plateau between 30 and 60 min later. All cells in fasted rats and all but one in non-fasted rats increased their activity after i.v insulin.

Recordings were made from 10 oxytocin cells in ten fasted rats and from 10 cells in nine non-fasted rats (including one double recording). In non-fasted rats, the mean (range) basal firing rate of 2.5(0.7-4.1)±0.4 spikes/s increased by 0.9(0.1-2.5)±0.3 spikes/s (averaged over the 60 min after i.v. insulin; P=0.002, Wilcoxon signed-rank test; Fig.2A,B). In fasted rats, oxytocin cells responded more strongly (Fig.2C): the basal firing rate of 2.4 (0.6-4.8)±0.5 spikes/s increased by 1.6(0.4-2.7)±0.3 spikes/s (P=0.002, Wilcoxon signed-rank test; P=0.045 for comparison of fasted and non-fasted rats, Mann-Whitney U test).

Vasopressin cells

In six fasted rats, recordings were made from ten vasopressin cells (three phasic- and seven continuously firing) with a mean (range) basal firing rate of 5.0 (0.2-8.1)±0.8 spikes/s. After insulin, the rate increased by 1.0 (-0.2-3.5)±0.3 spikes/s over 60 min (P=0.006, Wilcoxon signed-rank test; Fig.2D). In the three phasic cells, insulin increased the burst duration from 32±11 s to 82±29 s, and decreased the interburst duration from 32±15 s to 28±15 s; the resulting activity quotient increased from 0.5±0.2 to 0.7±0.2. The intraburst frequency was unchanged (basal: 3.6±0.7 spikes/s, insulin: 3.6±0.9 spikes/s). These changes were not statistically significant.

In 14 non-fasted rats, recordings were made from 16 vasopressin cells (eight phasic, eight continuous) with a basal firing rate of 5.1(1.4-10.1)±0.7 spikes/s. After insulin, the rate increased by 0.7(-0.7-2.8)±0.3 spikes/s over 60 min (P=0.028, Wilcoxon signed-rank test; Fig. 2D). In the eight phasic cells, insulin increased the burst duration (from 73±17 s, to 328±137 s). In these cells, the interburst period was reduced (from 64±26 to 61±18 s); the activity quotient was increased from 0.6±0.1 to 0.7±0.1, and the intraburst frequency was increased from 6.6±0.8 to 7.2±0.7 spikes/s.

Eight of ten vasopressin cells in fasted rats and nine of sixteen vasopressin cells in non-fasted rats increased their activity by more than 10% after i.v. insulin, and the mean response of all vasopressin cells tested was greater in fasted rats than in non-fasted rats, but this did not reach statistical significance (Mann-Whitney U test, P=0.63).

Hazard functions

In oxytocin cells, the hazard functions conformed to the profile previously reported as typical of oxytocin cells, reflecting a prolonged post-spike refractoriness of 30-50 ms followed by a stable plateau of excitability(42). Insulin did not affect the duration of the post-spike refractoriness but elevated the plateau level of excitability (Fig. 2E).

In vasopressin cells, the hazard functions also conformed to the profile previously reported as typical of vasopressin cells, reflecting a post-spike refractoriness of 20-50 ms followed by a period of hyperexcitability (reflecting a depolarising afterpotential) before reaching a stable plateau of excitability(42). Insulin did not affect the duration of the post-spike refractoriness or the plateau level of excitability but enhanced the post-spike hyperexcitability (Fig.2F).

Effect of i.v. insulin on blood glucose and spike activity of SON neurones

At the time of recording from SON neurones (~ 3h after i.p. anaesthesia), blood glucose concentrations were 12.7±0.8 mmol/l in fasted rats and 19.9±2.0 mmol/l in non-fasted rats. In both groups, plasma glucose levels were unchanged after injections of vehicle (0.9% saline) but fell after i.v. insulin, reaching a nadir after 60 min (Fig.3A; Two-way ANOVA; interaction $F(18, 162) = 6.69$; time, $F(6, 162) = 20.11$; treatment, $F(3, 27) = 10.8$; subject, $F(27, 162) = 12.9$, all $P<0.001$).

To test whether the activation of SON neurones by i.v insulin reflected the reduction in plasma glucose concentrations, we injected insulin to activate SON neurones in ten fasted

rats and then gave i.v. glucose to restore basal glucose concentrations (Fig.3B; one-way ANOVA, F (1.936, 17.42) = 9.275 P=0.002).

We tested five oxytocin cells in four of these rats. The mean basal firing rate (1.9(0.3-4.4)±0.7 spikes/s) increased by 1.1(0.4-1.7)±0.2 spikes/s after insulin. After i.v. glucose, the firing rate continued to increase reaching a final mean change of 1.6(1.0-3.0)±0.4 spikes/s (Fig.3C; Wilcoxon matched-pairs signed rank test, P=0.06).

We tested six vasopressin cells (of which one fired phasically) in six of the rats (one of the vasopressin cells was recorded simultaneously with an oxytocin cell). The mean basal firing rate (5.2(3.4-6.9)±0.6 spikes/s) increased by 1.2(0.6-2.4)±0.3 spikes/s after insulin. After glucose, the rate did not change significantly (final mean change 1.3(0.6-2.3)±0.3 spikes/s; Fig.3D; Wilcoxon matched-pairs signed rank test, P=0.8).

Thus, in the case of both oxytocin cells and vasopressin cells, responses to insulin were unaffected by i.v injections of glucose.

Blockade of central InsRs before i.v. insulin

To test whether the activation of SON neurones by insulin involves brain InsRs, we studied the effect of central administration of S961 on the responses (Fig.4). At 30-min after insulin injection, the blood glucose concentration in rats pretreated with i.c.v. S961 had fallen by 7.4±1.0 mmol/l, similar to the fall in fasted rats injected with insulin alone (7.4±0.9 mmol/l), indicating that the central injection of S961had no peripheral effect within this time (Fig.4C; repeated measures ANOVA, F(2, 28) = 49.8; P<0.0001).

The firing rate of eight oxytocin cells (2.8(1.3-5.3)±0.5 spikes/s; from 8 rats) was not significantly affected by S961 (change 0.2(-0.7-1.3)±0.2 spikes/s; Wilcoxon signed rank test, P=0.54). However, i.v. insulin had no effect in rats pretreated with S961 (change: -0.01(-0.4-0.3)±0.1 spikes/s after i.v. insulin; Wilcoxon signed rank test, P=1.0, Fig.4A).

Similarly the firing rate of eleven vasopressin cells (5.7(3.7-7.7)±0.3 spikes/s; 6 continuous- and 5 phasic cells in 10 rats) was not significantly affected by S961 (change 0.16 (-2.3-1.7)±0.3 spikes/s; Wilcoxon signed rank test, P=0.37). After i.v. insulin, their firing rate increased by 0.5(-1.5-3.3)±0.4 spikes/s, but this was not significant (Wilcoxon signed rank test, P=0.24; Fig.4B).

Thus central administration of S961 blocked the responsiveness of oxytocin cells to systemic administration of insulin but had no significant effect on vasopressin cells**.**

Effect of blockade of central InsRs on oxytocin spike activity induced by SCM gavage

Gavage of food rich in sugars, but not fat, results in a rise of blood glucose and insulin plasma concentration and a progressive increase in the electrical activity of oxytocin cells (37). Here, we tested whether this involves brain InsRs.

Both vehicle- and S961-injected rats exhibited a significant increase in both blood glucose concentration and plasma insulin concentration following SCM gavage (Fig.5A) with no significant differences between groups (glucose: two way-ANOVA for repeated measures: interaction, $F(3, 24) = 0.7769$, $P=0.52$; time, $F(3, 24) = 44.41$, $P < 0.0001$; treatment, $F(1, 8) = 0.4899$, $P = 0.5038$; subjects, $F(8, 24) = 29.9$, $P < 0.0001$; insulin: interaction, $F(3, 18) = 0.083$, $P=0.97$; interaction, $F(3, 18) = 10.17$, $P=0.0004$; treatment, $F(1, 6) = 0.18$, $P=0.7$; subjects, $F(6, 18) = 2.1$, $P=0.1$).

In vehicle-injected rats, as expected (37), SON oxytocin cells were progressively activated during SCM gavage. The firing rate of five oxytocin cells (from four rats) increased from 2.5 (1.3-3.1)±0.3 spikes/s.by 1.1(0.7-1.5)±0.1 spikes/s over during 60 min of gavage (Fig.5B). By contrast, in rats injected with S961, oxytocin cells did not respond to SCM gavage (Fig. 5C). The firing rate of five oxytocin cells (from five rats) increased from 3.2(1.4-5.4)±0.8 spikes/s by -0.1 (-1.0-0.7)±0.3 spikes/s during 60 min of gavage. This response was significantly different to the control group (*P=0.016 Mann Whitney test; Fig.5D,E).

DISCUSSION

Recently, the role of oxytocin cells in metabolic regulation has attracted increasing attention. Most attention has been given to the oxytocin cells of the PVN, as these include a small population of parvocellular neurones that project to the dorsal vagal complex and to the spinal cord, where their actions include effects on gastric reflexes, energy intake and thermogenesis (23, 43, 44). Until relatively recently, the magnocellular oxytocin cells, which comprise most of the oxytocin cells in the PVN and all those in the SON, were thought to have few central projections. However, these neurones, which all project axons to the posterior pituitary, also release large amounts of oxytocin within the brain from their dendrites. This dendritic release is likely to have important effects at relatively local sites,

including the amygdala and the ventromedial nucleus of the hypothalamus where abundant oxytocin receptors are expressed but which contain only sparse oxytocin fibres (23, 45). In addition, it has recently become apparent that many magnocellular neurones have extensive axonal projections to diverse brain regions, including notably to the nucleus accumbens.(46)

In the present study, systemic administration of insulin increased the electrical activity of both oxytocin and vasopressin SON cells, consistent with previous reports in humans and rats that insulin increases secretion of oxytocin and vasopressin (47-49).

As originally conceived in the design of the present experiments, the dose and route of insulin administration followed the conventional design of insulin tolerance tests (50), to produce an acute maintained hypoglycaemia. This bolus injection raises peripheral insulin concentrations above the normal physiological range, which are then rapidly cleared. The evolution of oxytocin cell activity after insulin injections thus mirrored neither the changes in plasma glucose nor the expected changes in peripheral insulin concentration. Insulin crosses the blood-brain barrier by an active transport mechanism that is saturated: at least 50% of maximal transport capacity is reached at euglycemic levels of plasma insulin, thus supraphysiological levels of insulin in the plasma have little additional effect on insulin penetration into the brain beyond that seen at high physiological levels (1, 51). Thus the expected evolution of CNS insulin following i.v. bolus injection is a progressive rise while peripheral levels are elevated above normal levels – possibly explaining the progressive rise in oxytocin cell activity.

Brain InsRs play an important role in the control of energy balance as shown by selective genetically-induced decreased expression of brain InsRs which is linked to a peripheral metabolic alterations, including increased food intake, fat, and body weight, as well as increased glucose and insulin resistance in rodents (52, 53). Moreover, injection of the InsR antagonist S961 into the ventromedial nucleus increases blood glucose concentration in rats (54). In the present study, central administration of S916 prevented the insulin-induced responses in all oxytocin cells, indicating that systemic insulin penetrates into the brain to activate SON neurones by actions on central InsRs. The central administration of S916 produced a non-significant increase in plasma glucose concentrations consistent with its reported effects in the ventromedial nucleus (54), and did not affect the effect of systemically applied insulin on plasma glucose levels, indicating that, at this dose and by this route, it did not block systemic effects of exogenous insulin.

In humans, glucose, but not fructose, infusion has been shown to prevent oxytocin and vasopressin release by insulin-induced hypoglycaemia (55).In this study, we infused bolus

injections of glucose solution to approximately clamp circulating glucose concentrations after i.v. insulin. Once the firing response was triggered, neither oxytocin nor vasopressin cells reduced their spike activity after glucose injections.

In non-fasted rats, which exhibited a more pronounced hyperglycaemia than fasted rats, the responses of oxytocin cells were less prominent than in fasted rats. This may reflect InsR desensitisation in oxytocin cells, similarly to that shown in skeletal muscle *in vivo* (56) and fibroblasts *in vitro* (57), where acute exposition to high glucose concentration reduced insulin-stimulated glucose uptake and impaired InsR intracellular signalling, respectively. Alternatively, as in fasted animals blood glucose concentrations fell following insulin administration to concentrations lower than immediately after anaesthesia, this might stimulate the hypothalamo-pituitary-adrenal (HPA) as occurs in the insulin tolerance test (55), potentiating the release of oxytocin (and vasopressin).

A recent study (17) raised the question about the capacity of SON neurones to respond to insulin administration since insulin given i.c.v. induced an increase in Fos expression after 90-min in 13 % of the PVN, but not SON, oxytocin cells compared to control mice. Nevertheless, SON neurones appear to be intrinsically sensitive to insulin and glucose as they express InsR (12-14) and also the enzyme glucokinase (58), a marker for glucose sensing. Moreover, vasopressin and oxytocin are released from SON explants in the presence of medium containing glucose and insulin (16). Although Fos protein has been widely used as a marker for neuronal activation, its lack of expression does not necessarily exclude changes in neural activity as observed in some conditions, and increased spike activity is not invariably linked to Fos expression (59, 60). It seems that insulin might not induce the expected rapid expression of Fos (i.e. 60-90 min) as Griffond et al. reported that at 1 h after insulin i.p. (20 mg/kg), there was little expression of Fos in PVN oxytocin cells(15).

A limitation of this study is that it involved urethane-anesthetised rats. Urethane has long been the anaesthetic of choice for SON electrophysiological recordings, as it provides a deep long-lasting stable anaesthesia compatible with transpharyngeal surgery without affecting the physiological responses of SON neurones (40). However, urethane raises blood glucose concentrations (61, 62) by increasing sympathetic tone (63) and consequently increasing gluconeogenesis. Thus, blood glucose concentrations in both non-fasted and fasted anaesthetised rats were higher than in conscious Sprague-Dawley rats (64). However they were lower in fasted rats than in non-fasted rats, and changed in the expected manner in response to i.v. insulin.

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Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author contributions

The study was designed by GL and performed by LP. LP and GL analysed the data and wrote the paper together. GL had full access to all the data and analyses, and takes responsibility for the integrity of the data and the accuracy of the analyses.

Figure 1. Identification of SON neurons. (A) Voltage trace showing the electrical stimulation (red stimulus artefact; 0 ms) of the pituitary stalk which evokes an antidromic spike travelling to the soma of SON neurones at a constant latency (~10 ms) in each of two neurons (red spikes), and spike collision occurring when an spontaneous orthodromically traveling spike (black spike) encounters an antidromic spike (centre and right panels) extinguishing it. (B) Raw voltage trace of a double recording showing the spike activity of a continuous- (black large spikes) and a phasic-firing (red short spikes exhibiting intermittent periods of activity) vasopressin cell. (C, D) Typical ISI histogram (frequency of time distributions between (two) occurring spikes) over 10-min and corresponding hazard function of the basal spontaneous activity of an (C) oxytocin cell, and (D) vasopressin cell. (E,F) Pharmacological identification of SON neurones. Raw voltage trace (upper panel) and firing rate (spikes in 10s bins; lower panel) of an (E) oxytocin, and (F) vasopressin cell exhibiting transient excitation and inhibition respectively in response to i.v. CCK (20 μg/kg).

Figure 2. Effect of i.v. insulin on SON neurones in fasted rats.

(A) Representative example of the firing rate of an identified oxytocin cell (in 30-s bins; upper panel and instantaneous frequency plot below) in response to an i.v. injection of insulin. The dashed line indicates the mean basal firing rate. (B) ISI distribution and average waveform (lower panels) over 10-min period during baseline (left, corresponding to the solid line in A) and maximal neuronal response (right, corresponding to the dotted line in A) of the neurone shown in A. (C). Average responses (mean changes from baseline ± SEM) of ten

oxytocin cells in fasted rats (black) and ten in non-fasted rats (white) to i.v. insulin. (D) Average responses of 16 vasopressin cells in fasted rats (black) and 16 in non-fasted rats (white) to i.v. insulin. (E) Mean (SEM) Hazard functions of the ten oxytocin cells in fasted rats before (closed symbols) and after insulin (open symbols). (F). Mean (SEM) Hazard functions of eight vasopressin cells in fasted rats before (closed symbols) and after insulin (open symbols; two cells excluded as the basal firing rates were too low to construct hazard functions).

Figure 3. Effect of i.v. glucose infusion in insulin-responsive neurones in fasted rats.
(A) Blood glucose concentrations were lowered after i.v. insulin, but not i.v. vehicle, in fasted and non-fasted rats (*P < 0.05, Two-way ANOVA followed by Bonferroni *post hoc* test). (B) Blood glucose concentrations after i.v. insulin and after i.v. insulin, and 5% glucose solution injections (arrows: as required) of all 10 rats where neuronal activity was recorded. After 30-min of i.v. insulin, the glucose concentration was significantly lower compared to all other blood samples (one-way ANOVA for repeated measures; ***P<0.001, Bonferroni *post hoc*) with no significant differences between other samples. (B) Blood glucose concentrations after i.v. insulin, and 5% glucose solution injections (arrows: 400 µl, *300 µl, *100 µl; * if required) of all animals (n=10) where neural activity was recorded. (C,D) After insulin, no significant differences in firing rate of (C) oxytocin, and (D) vasopressin cells in glucose-treated rats were detected when compared to non-glucose treated fasted rats. Data are means ± SEM.

Figure 4. Effect of central InsR blockade on neuronal responses following i.v. insulin in fasted rats.
(A,B) Administration of S961 (1 nmole i.c.v.) blocked the increase in firing rate (in 10-min bins) induced by i.v. insulin in (A) oxytocin, but not (B) vasopressin cells when compared to fasted rats injected with i.v. insulin alone (Figure 3). (C) Blood glucose concentration tended (P = 0.13) to increase 15 min after of i.c.v. InsR antagonist administration, insulin i.v. significantly lowered the blood glucose concentration after 30-min compared to basal, and InsR antagonist blood samples (***P<0.001, One-way ANOVA for repeated measurements, followed by Bonferroni *post hoc* test*;* n=15), in brackets, times since basal. Data are means ± SEM.

Figure 5. Effect of central InsR blockade on SCM-induced increase in firing rate in oxytocin cells.

(A) Gavage of 5 ml of SCM significantly increased blood glucose concentrations (left panel; $P<0.05$, Two-way ANOVA followed by Bonferroni post hoc compared to baseline) and plasma insulin (right panel) in both i.c.v. vehicle- and S961-treated rats with no significant differences between groups. (B) Representative examples showing the increase in firing rate (in 30-s bins) induced by SCM gavage (5 ml) in a vehicle-injected rat (upper panel), and blockade of SCM-induced response in an. S961-injected rat. (C) Mean change (± S.E.M.) in firing rate over 60 min from all oxytocin cells recorded in i.c.v. vehicle- and S961-injected rats gavaged with SCM (*$P<0.05$ Mann-Whitney test). (D) The change in firing rate (in 10-min bins) was significantly different between groups after 30-min since the beginning of SCM gavage. Data are means ± SEM.

References

1. Banks WA, Jaspan JB, Huang W, Kastin AJ. Transport of insulin across the blood-brain barrier: saturability at euglycemic doses of insulin. *Peptides*. 1997; **18**(9): 1423-9.
2. Baura GD, Foster DM, Porte D, Jr., Kahn SE, Bergman RN, Cobelli C, Schwartz MW. Saturable transport of insulin from plasma into the central nervous system of dogs in vivo. A mechanism for regulated insulin delivery to the brain. *The Journal of clinical investigation*. 1993; **92**(4): 1824-30.
3. Strubbe JH, Porte D, Jr., Woods SC. Insulin responses and glucose levels in plasma and cerebrospinal fluid during fasting and refeeding in the rat. *Physiology & behavior*. 1988; **44**(2): 205-8.
4. Wallum BJ, Taborsky GJ, Jr., Porte D, Jr., Figlewicz DP, Jacobson L, Beard JC, Ward WK, Dorsa D. Cerebrospinal fluid insulin levels increase during intravenous insulin infusions in man. *The Journal of clinical endocrinology and metabolism*. 1987; **64**(1): 190-4.
5. Woods SC, Porte D, Jr. Relationship between plasma and cerebrospinal fluid insulin levels of dogs. *The American journal of physiology*. 1977; **233**(4): E331-4.
6. Steffens AB, Scheurink AJ, Porte D, Jr., Woods SC. Penetration of peripheral glucose and insulin into cerebrospinal fluid in rats. *The American journal of physiology*. 1988; **255**(2 Pt 2): R200-4.
7. Kullmann S, Heni M, Fritsche A, Preissl H. Insulin action in the human brain: evidence from neuroimaging studies. *Journal of neuroendocrinology*. 2015; **27**(6): 419-23.
8. Gray SM, Meijer RI, Barrett EJ. Insulin regulates brain function, but how does it get there? *Diabetes*. 2014; **63**(12): 3992-7.
9. Niswender KD, Morrison CD, Clegg DJ, Olson R, Baskin DG, Myers MG, Jr., Seeley RJ, Schwartz MW. Insulin activation of phosphatidylinositol 3-kinase in the hypothalamic arcuate nucleus: a key mediator of insulin-induced anorexia. *Diabetes*. 2003; **52**(2): 227-31.
10. Sohn JW, Oh Y, Kim KW, Lee S, Williams KW, Elmquist JK. Leptin and insulin engage specific PI3K subunits in hypothalamic SF1 neurons. *Molecular metabolism*. 2016; **5**(8): 669-79.
11. Qiu J, Wagner EJ, Ronnekleiv OK, Kelly MJ. Insulin and leptin excite anorexigenic pro-opiomelanocortin neurones via activation of TRPC5 channels. *Journal of neuroendocrinology*. 2018; **30**(2).
12. Unger J, McNeill TH, Moxley RT, 3rd, White M, Moss A, Livingston JN. Distribution of insulin receptor-like immunoreactivity in the rat forebrain. *Neuroscience*. 1989; **31**(1): 143-57.
13. Hill JM, Lesniak MA, Pert CB, Roth J. Autoradiographic localization of insulin receptors in rat brain: prominence in olfactory and limbic areas. *Neuroscience*. 1986; **17**(4): 1127-38.

14. Corp ES, Woods SC, Porte D, Jr., Dorsa DM, Figlewicz DP, Baskin DG. Localization of 125I-insulin binding sites in the rat hypothalamus by quantitative autoradiography. *Neuroscience letters*. 1986; **70**(1): 17-22.
15. Griffond B, Deray A, Bahjaoui-Bouhaddi M, Colard C, Bugnon C, Fellmann D. Induction of Fos-like immunoreactivity in rat oxytocin neurons following insulin injections. *Neuroscience letters*. 1994; **178**(1): 119-23.
16. Song Z, Levin BE, Stevens W, Sladek CD. Supraoptic oxytocin and vasopressin neurons function as glucose and metabolic sensors. *American journal of physiology Regulatory, integrative and comparative physiology*. 2014; **306**(7): R447-56.
17. Zhang B, Nakata M, Nakae J, Ogawa W, Yada T. Central insulin action induces activation of paraventricular oxytocin neurons to release oxytocin into circulation. *Scientific reports*. 2018; **8**(1): 10415.
18. Augustine RA, Seymour AJ, Campbell RE, Grattan DR, Brown CH. Integrative neuro-humoral regulation of oxytocin neuron activity in pregnancy and lactation. *Journal of neuroendocrinology*. 2018.
19. Olazabal DE. Role of oxytocin in parental behaviour. *Journal of neuroendocrinology*. 2018; **30**(7): e12594.
20. Neumann ID, Landgraf R. Tracking oxytocin functions in the rodent brain during the last 30 years: From push-pull perfusion to chemogenetic silencing. *Journal of neuroendocrinology*. 2019; **31**(3): e12695.
21. Leng G, Russell JA. The osmoresponsiveness of oxytocin and vasopressin neurones: Mechanisms, allostasis and evolution. *Journal of neuroendocrinology*. 2019; **31**(3): e12662.
22. Onaka T, Takayanagi Y. Role of oxytocin in the control of stress and food intake. *Journal of neuroendocrinology*. 2019; **31**(3): e12700.
23. Leng G, Sabatier N. Oxytocin - The Sweet Hormone? *Trends in endocrinology and metabolism: TEM*. 2017; **28**(5): 365-76.
24. Ho JM, Blevins JE. Coming full circle: contributions of central and peripheral oxytocin actions to energy balance. *Endocrinology*. 2013; **154**(2): 589-96.
25. Blevins JE, Graham JL, Morton GJ, Bales KL, Schwartz MW, Baskin DG, Havel PJ. Chronic oxytocin administration inhibits food intake, increases energy expenditure, and produces weight loss in fructose-fed obese rhesus monkeys. *American journal of physiology Regulatory, integrative and comparative physiology*. 2015; **308**(5): R431-8.
26. Leslie M, Leppanen J, Paloyelis Y, Treasure J. The influence of oxytocin on eating behaviours and stress in women with bulimia nervosa and binge eating disorder. *Mol Cell Endocrinol*. 2018110354.
27. Kaneto A, Kosaka K, Nakao K. Effects of the neurohypophysial hormones on insulin secretion. *Endocrinology*. 1967; **81**(4): 783-90.
28. Oshikawa S, Tanoue A, Koshimizu TA, Kitagawa Y, Tsujimoto G. Vasopressin stimulates insulin release from islet cells through V1b receptors: a combined pharmacological/knockout approach. *Molecular pharmacology*. 2004; **65**(3): 623-9.
29. Mineo H, Ito M, Muto H, Kamita H, Hyun HS, Onaga T, Yanaihara N. Effects of oxytocin, arginine-vasopressin and lysine-vasopressin on insulin and glucagon secretion in sheep. *Research in veterinary science*. 1997; **62**(2): 105-10.
30. Stock S, Fastbom J, Bjorkstrand E, Ungerstedt U, Uvnas-Moberg K. Effects of oxytocin on in vivo release of insulin and glucagon studied by microdialysis in the rat pancreas and autoradiographic evidence for [3H]oxytocin binding sites within the islets of Langerhans. *Regulatory peptides*. 1990; **30**(1): 1-13.
31. Ott V, Finlayson G, Lehnert H, Heitmann B, Heinrichs M, Born J, Hallschmid M. Oxytocin reduces reward-driven food intake in humans. *Diabetes*. 2013; **62**(10): 3418-25.

32. Klement J, Ott V, Rapp K, Brede S, Piccinini F, Cobelli C, Lehnert H, Hallschmid M. Oxytocin Improves beta-Cell Responsivity and Glucose Tolerance in Healthy Men. *Diabetes*. 2017; **66**(2): 264-71.
33. Leng G, Sabatier N. Measuring Oxytocin and Vasopressin: Bioassays, Immunoassays and Random Numbers. *Journal of neuroendocrinology*. 2016; **28**(10).
34. Szulc P, Amri EZ, Varennes A, Panaia-Ferrari P, Fontas E, Goudable J, Chapurlat R, Breuil V. High serum oxytocin is associated with metabolic syndrome in older men - The MINOS study. *Diabetes Res Clin Pract*. 2016; **122**17-27.
35. Enhorning S, Wang TJ, Nilsson PM, Almgren P, Hedblad B, Berglund G, Struck J, Morgenthaler NG, Bergmann A, Lindholm E, Groop L, Lyssenko V, Orho-Melander M, Newton-Cheh C, Melander O. Plasma copeptin and the risk of diabetes mellitus. *Circulation*. 2010; **121**(19): 2102-8.
36. Zerbe RL, Vinicor F, Robertson GL. Plasma vasopressin in uncontrolled diabetes mellitus. *Diabetes*. 1979; **28**(5): 503-8.
37. Hume C, Sabatier N, Menzies J. High-Sugar, but Not High-Fat, Food Activates Supraoptic Nucleus Neurons in the Male Rat. *Endocrinology*. 2017; **158**(7): 2200-11.
38. Schaffer L, Brand CL, Hansen BF, Ribel U, Shaw AC, Slaaby R, Sturis J. A novel high-affinity peptide antagonist to the insulin receptor. *Biochemical and biophysical research communications*. 2008; **376**(2): 380-3.
39. Shi Z, Hansen KM, Bullock KM, Morofuji Y, Banks WA, Brooks VL. Resistance to the sympathoexcitatory effects of insulin and leptin in late pregnant rats. *The Journal of physiology*. 2019; **597**(15): 4087-100.
40. Leng G, Sabatier N. Electrophysiology of Magnocellular Neurons in Vivo. In: Armstrong WE, Tasker JG, eds. *Neurophysiology of Neuroendocrine Neurons*. Chichester, UK: John Wiley & Sons, Ltd. 2014: 1-28.
41. Brown CH, Bains JS, Ludwig M, Stern JE. Physiological regulation of magnocellular neurosecretory cell activity: integration of intrinsic, local and afferent mechanisms. *Journal of neuroendocrinology*. 2013; **25**(8): 678-710.
42. Sabatier N, Brown CH, Ludwig M, Leng G. Phasic spike patterning in rat supraoptic neurones in vivo and in vitro. *The Journal of physiology*. 2004; **558**(Pt 1): 161-80.
43. Ong ZY, Bongiorno DM, Hernando MA, Grill HJ. Effects of Endogenous Oxytocin Receptor Signaling in Nucleus Tractus Solitarius on Satiation-Mediated Feeding and Thermogenic Control in Male Rats. *Endocrinology*. 2017; **158**(9): 2826-36.
44. Roberts ZS, Wolden-Hanson T, Matsen ME, Ryu V, Vaughan CH, Graham JL, Havel PJ, Chukri DW, Schwartz MW, Morton GJ, Blevins JE. Chronic hindbrain administration of oxytocin is sufficient to elicit weight loss in diet-induced obese rats. *American journal of physiology Regulatory, integrative and comparative physiology*. 2017; **313**(4): R357-R71.
45. Chini B, Verhage M, Grinevich V. The Action Radius of Oxytocin Release in the Mammalian CNS: From Single Vesicles to Behavior. *Trends Pharmacol Sci*. 2017; **38**(11): 982-91.
46. Althammer F, Grinevich V. Diversity of oxytocin neurons: beyond magno- and parvocellular cell types? *Journal of neuroendocrinology*. 2017.
47. Fisher BM, Baylis PH, Frier BM. Plasma oxytocin, arginine vasopressin and atrial natriuretic peptide responses to insulin-induced hypoglycaemia in man. *Clinical endocrinology*. 1987; **26**(2): 179-85.
48. Baylis PH, Robertson GL. Rat vasopressin response to insulin-induced hypoglycemia. *Endocrinology*. 1980; **107**(6): 1975-9.
49. Bjorkstrand E, Eriksson M, Uvnas-Moberg K. Plasma levels of oxytocin after food deprivation and hypoglycaemia, and effects of 1-deamino-2-D-Tyr-(OEt)-4-Thr-8-Orn-oxytocin on blood glucose in rats. *Acta physiologica Scandinavica*. 1992; **144**(3): 355-9.
50. Kinzig KP, Honors MA, Hargrave SL. Insulin sensitivity and glucose tolerance are altered by maintenance on a ketogenic diet. *Endocrinology*. 2010; **151**(7): 3105-14.

51. Banks WA, Owen JB, Erickson MA. Insulin in the brain: there and back again. *Pharmacol Ther*. 2012; **136**(1): 82-93.
52. Obici S, Feng Z, Karkanias G, Baskin DG, Rossetti L. Decreasing hypothalamic insulin receptors causes hyperphagia and insulin resistance in rats. *Nature neuroscience*. 2002; **5**(6): 566-72.
53. Bruning JC, Gautam D, Burks DJ, Gillette J, Schubert M, Orban PC, Klein R, Krone W, Muller-Wieland D, Kahn CR. Role of brain insulin receptor in control of body weight and reproduction. *Science*. 2000; **289**(5487): 2122-5.
54. Paranjape SA, Chan O, Zhu W, Horblitt AM, McNay EC, Cresswell JA, Bogan JS, McCrimmon RJ, Sherwin RS. Influence of insulin in the ventromedial hypothalamus on pancreatic glucagon secretion in vivo. *Diabetes*. 2010; **59**(6): 1521-7.
55. Chiodera P, Volpi R, Capretti L, Speroni G, Marcato A, Rossi G, Coiro V. Hypoglycemia-induced arginine vasopressin and oxytocin release is mediated by glucoreceptors located inside the blood-brain barrier. *Neuroendocrinology*. 1992; **55**(6): 655-9.
56. Hoy AJ, Bruce CR, Cederberg A, Turner N, James DE, Cooney GJ, Kraegen EW. Glucose infusion causes insulin resistance in skeletal muscle of rats without changes in Akt and AS160 phosphorylation. *American journal of physiology Endocrinology and metabolism*. 2007; **293**(5): E1358-64.
57. Pillay TS, Xiao S, Olefsky JM. Glucose-induced phosphorylation of the insulin receptor. Functional effects and characterization of phosphorylation sites. *The Journal of clinical investigation*. 1996; **97**(3): 613-20.
58. Navarro M, Rodriquez de Fonseca F, Alvarez E, Chowen JA, Zueco JA, Gomez R, Eng J, Blazquez E. Colocalization of glucagon-like peptide-1 (GLP-1) receptors, glucose transporter GLUT-2, and glucokinase mRNAs in rat hypothalamic cells: evidence for a role of GLP-1 receptor agonists as an inhibitory signal for food and water intake. *Journal of neurochemistry*. 1996; **67**(5): 1982-91.
59. Luckman SM, Dyball RE, Leng G. Induction of c-fos expression in hypothalamic magnocellular neurons requires synaptic activation and not simply increased spike activity. *J Neurosci*. 1994; **14**(8): 4825-30.
60. Sabatier N, Caquineau C, Dayanithi G, Bull P, Douglas AJ, Guan XM, Jiang M, Van der Ploeg L, Leng G. Alpha-melanocyte-stimulating hormone stimulates oxytocin release from the dendrites of hypothalamic neurons while inhibiting oxytocin release from their terminals in the neurohypophysis. *J Neurosci*. 2003; **23**(32): 10351-8.
61. Reinert H. Urethane Hyperglycaemia and Hypothalamic Activation. *Nature*. 1964; **204**889-91.
62. Wang MY, Ren LM, Du ZJ, Fu SX. Urethane-induced hyperglycemia. *Acta pharmacologica Sinica*. 2000; **21**(3): 271-5.
63. Maggi CA, Meli A. Suitability of urethane anesthesia for physiopharmacological investigations in various systems. Part 1: General considerations. *Experientia*. 1986; **42**(2): 109-14.
64. Archer ZA, Rayner DV, Rozman J, Klingenspor M, Mercer JG. Normal distribution of body weight gain in male Sprague-Dawley rats fed a high-energy diet. *Obesity research*. 2003; **11**(11): 1376-83.