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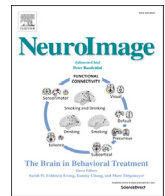


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Modulation of midbrain neurocircuitry by intranasal insulin

Sharmili Edwin Thanarajah^{a,b}, Sandra Iglesias^c, Bojana Kuzmanovic^a, Lionel Rigoux^a,
Klaas E. Stephan^{a,c}, Jens C. Brüning^{a,d}, Marc Tittgemeyer^{a,e,f,*}

^a Max-Planck-Institute for Metabolism Research, Cologne, Germany

^b Department of Neurology, University Hospital of Cologne, Cologne, Germany

^c Translational Neuromodeling Unit, Institute for Biomedical Engineering, University of Zurich and Swiss Federal Institute of Technology, Zurich, Switzerland

^d Center for Endocrinology, Diabetes and Preventive Medicine (CEDP), University Hospital of Cologne, Cologne, Germany

^e Modern Diet and Physiology Center, USA

^f Cologne Cluster of Excellence in Cellular Stress and Aging-Associated Disease (CECAD), Cologne, Germany

ABSTRACT

Insulin modulates dopamine neuron activity in midbrain and affects processes underlying food intake behaviour, including impulsivity and reward processing. Here, we used intranasal administration and task-free functional MRI in humans to assess time- and dose-dependent effects of insulin on functional connectivity of the dopaminergic midbrain – and how these effects varied depending on systemic insulin sensitivity as measured by HOMA-IR. Specifically, we used a repeated-measures design with factors dose (placebo, 40 IU, 100 IU, 160 IU), time (7 time points during a 90 min post-intervention interval), and group (low vs. high HOMA-IR).

A factorial analysis identified a three-way interaction (with whole-brain significance) with regard to functional connectivity between midbrain and the ventromedial prefrontal cortex. This interaction demonstrates that systemic insulin sensitivity modulates the temporal course and dose-dependent effects of intranasal insulin on midbrain functional connectivity. It suggests that altered insulin sensitivity may impact on dopaminergic projections of the midbrain and might underlie the dysregulation of reward-related and motivational behaviour in obesity and diabetes. Perhaps most importantly, the time courses of midbrain functional connectivity we present may provide useful guidance for the design of future human studies that utilize intranasal insulin administration.

1. Introduction

Most previous studies on the neural control of feeding behaviour focused on signalling mechanisms associated with the regulation of body weight homeostasis. Apart from impaired cerebral regulation of metabolic homeostasis, additional cognitive and affective factors can influence eating behaviour and induce excessive weight gain (Begg and Woods, 2013; Ferrario et al., 2016; Medic et al., 2016). In particular, observations that food anticipation and intake trigger dopaminergic responses that are modulated by diet and body weight (de Araujo et al., 2008; Medic et al., 2014; Stice et al., 2009, 2010; Wang et al., 2014) raise the question how homeostatic signals interact with dopaminergic neurocircuitry that is involved, amongst other processes, in reward processing and impulsivity (Buckholtz et al., 2010; Kenny, 2011b; Volkow et al., 2011).

Insulin is a key candidate for mediating this interaction. It represents a critical hormone for metabolic regulation but also impacts on dopamine-dependent processes (Lockie and Andrews, 2013): both hyperinsulinemia and insulin resistance have been associated with maladaptive eating behaviour (Heni et al., 2015; Kleinridders et al., 2015;

Könner and Brüning, 2012; Morton et al., 2014) and brain disorders involving dopaminergic transmission (Athauda and Foltynie, 2016). As a consequence, the question how insulin may regulate dopaminergic transmission is increasingly moving into the focus of studies on metabolic control.

Insulin receptors (IR) are expressed by neurons in numerous brain regions (Kleinridders et al., 2014; Plum et al., 2005). Remarkably, the major sources of dopaminergic (DA) neurons – the ventral tegmental area (VTA) and the substantia nigra (SN) in the midbrain (Morales and Margolis, 2017) – are particularly enriched in insulin receptors (IR, Figlewicz et al., 2003). Here, the IR is co-expressed with tyrosine hydroxylase (Th), a key enzyme and marker for catecholaminergic neurons. Th neuron-specific deletion of the IR was shown to result in a reduction of excitatory input on DA VTA/SN neurons (Figlewicz et al., 2003). Furthermore, Könner et al. (2011) demonstrated that insulin has a significant excitatory effect on a major subpopulation of DA VTA/SN neurons: it increases the firing rate of these DA neurons but also upregulates DA clearance via increased trafficking of DA reuptake transporters (DAT; Mebel et al., 2012) and higher DAT-mRNA expression (Figlewicz et al., 2007). This increased insulin-mediated DA clearance may contribute to

* Corresponding author. Max-Planck-Institute for Metabolism Research, Gleueler Str. 50, 50931 Cologne, Germany.

E-mail address: tittgemeyer@sf.mpg.de (M. Tittgemeyer).

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enhanced DA neuron excitability via reduced activation of inhibitory D2 autoreceptors on DA VTA/SN cells (Uchida et al., 2000). Finally, insulin induces long-term depression of excitatory synapses on VTA dopamine neurons by endocannabinoid signalling (Labouebe et al., 2013), a mechanism that is attenuated by hyperinsulinemia (Liu et al., 2013).

This set of insulin actions on DA neurons provides a potential link between the control of food intake and processes associated with dopaminergic circuitry and transmission (Figlewicz et al., 2007; Figlewicz and Sipols, 2010; Palmiter, 2007). For example, insulin modulates reward seeking and drug relapse – behaviours associated with DA signalling in the mesolimbic dopamine system (Davis et al., 2010; Figlewicz and Benoit, 2009; Kenny, 2011a). Furthermore, direct insulin application in the VTA reduces intracranial self-stimulation and food intake (Brujinzeel et al., 2011), and the consumption of sweetened high fat food can be decreased by insulin in the VTA (Mebel et al., 2012). In summary, numerous preclinical studies suggest that insulin action on DA neurons contributes to energy maintenance and consumption behaviour by adjusting the reward value of food items (Figlewicz et al., 2007). Interestingly, Brujinzeel et al. (2011) found that this effect is dose-dependent. While low doses of insulin in the VTA modulated the reward threshold, higher doses reduced food intake but did not change reward-related behaviour.

Despite these exciting findings, much remains to be learned about the detailed mechanism(s) of how insulin controls DA neuron excitability, and importantly, how insulin affects the dopaminergic circuitry in humans. Clearly, elucidating these mechanisms in detail is of great importance, both for understanding the role of the DA system in the development of obesity – e.g., body mass index is negatively correlated with dopamine receptor D2 (D2R) density in striatal regions (Volkow et al., 2008; Wang et al., 2001) – and for understanding how interactions between DA and insulin modulate non-homeostatic eating behaviour in general. Furthermore, given that interactions between mechanisms amplify the variability of jointly affected outcomes, the interaction between hormonal and neuromodulatory mechanisms may be key to understanding individual variability in eating behaviour and suggest new therapeutic strategies against obesity.

In humans, intranasal administration is the method of choice to study insulin effects on neuronal processes without relevant systemic side effects. Initial studies demonstrated a wide range of functional consequences from intranasal insulin application in humans (cf. Suppl. Table S1, for a collection of currently available reports), including reduced food intake both after single administration (Hallschmid et al., 2012; Jauch-Chara et al., 2012) and long-term application (Hallschmid et al., 2004). In line with animal studies, postprandial application in humans reduced the consumption of palatable food and increased satiety (Hallschmid et al., 2012). Functional magnetic resonance imaging studies (fMRI) investigated insulin effects on the response to food cues or at rest, showing modulation of neural activity in brain areas typically associated with food intake and reward behaviour (Guthoff et al., 2010; Kullmann et al., 2013, 2015; Schilling et al., 2014; Tiedemann et al., 2017; Zhang et al., 2015). In addition, a recent genetic imaging study suggested a relevant interaction between intranasally applied insulin and D2R availability (Heni et al., 2016).

These initial findings are not easily interpretable, since we lack knowledge about the pharmacokinetics of intranasally applied insulin in general and about the time course and dose-dependency of its modulatory effect on the midbrain in particular. So far, the only study on the pharmacokinetics of intranasally applied insulin used a single dose (40 I.U., Born et al., 2002) and was conducted invasively (via lumbar puncture). This study found peak insulin levels in the cerebrospinal fluid (CSF) 30 min after intranasal insulin application. However, peak insulin accumulation in the CSF does not correspond to peak neuronal effects, nor does it provide information about insulin action on midbrain activity or connectivity. Relying on the report by Born et al. (2002), most recent studies tested central effects of intranasal insulin 30 min after administration of 40 I.U. or 160 I.U. (Suppl. Table S1). It remains unclear,

however, which dose is effective to modulate dopaminergic processes and at which time point after intranasal application neurons respond.

To fill this gap of knowledge and assess the time- and dose-dependent effects of insulin on dopaminergic transmission, we designed a randomized, placebo-controlled pharmacological fMRI study with a within-subject (crossover) design. We investigated the effect of intranasal administration of insulin under three commonly used doses (40, 100 and 160 I.U.), taking six measurements during a time period of 15–90 min after application. As a proxy for a readout of dopaminergic transmission, we measured the functional connectivity of the dopaminergic midbrain (VTA/SN) with the rest of the brain. Given that insulin in the brain influences food intake and body weight in lean persons (Heni et al., 2015) and no such effect has yet been observed in overweight individuals (Hallschmid et al., 2008; Heni et al., 2017), we predicted that insulin-dopamine interactions may change with BMI. We therefore examined two groups, i.e., individuals with normal weight and overweight. Interestingly, we could not find an insulin-dopamine interaction with BMI. Hence, we analysed the insulin-dopamine interaction with peripheral insulin sensitivity assessed by the homeostasis model assessment of insulin resistance (HOMA-IR, McAuley et al., 2007). It is important to note that none of these subjects were obese or suffered from diabetes.

2. Materials and methods

Participants. Twenty-one subjects of normal weight ($22.4 \text{ kg/m}^2 \pm 1.7$) as well as twenty-one overweight subjects ($28.0 \text{ kg/m}^2 \pm 4.0$) were recruited from the pre-existing database of volunteers maintained at the Max-Planck-Institute for Metabolic Research. All participants were medication-free and male. We only employed male participants to exclude variations of hormonal effects on the BOLD signal during the menstrual cycle. All participants were non-smokers without any history of neurological, psychiatric, gastrointestinal or eating disorders, and without any special diets or medical treatments. To exclude athletes whose BMI would, due to increased muscle mass, falsely classify them as overweight, we included only participants who indicated that they do not engage in high-intensity physical workout. In the course of data analysis, six subjects had to be excluded: three subjects due to malfunction of the MR scanner, one showed strong motion artefacts in the placebo condition, one had a significantly enhanced cortisol level during the baseline condition of the placebo day compared to all other testing days and one subject significantly lost weight (3.4 kg) during the study. In total, 17 lean (BMI: $22.9 \text{ kg/m}^2 \pm 1.5$, age: $27.0 \text{ yrs} \pm 4.0$) and 19 overweight subjects ($27.2 \text{ kg/m}^2 \pm 1.5$, age: $30.0 \text{ yrs} \pm 4.9$) were included in further data analyses (Table 1). After analysing the effects of group stratified by body weight (normal vs. overweight), we considered to scrutinize how insulin-dopamine interactions in the brain may change with systemic insulin resistance. Hence, subjects were classified for the analysis into two groups, i.e., individuals with higher and lower peripheral insulin sensitivity assessed by the homeostasis model assessment of insulin resistance (HOMA-IR, Matthews et al., 1985; McAuley et al., 2007). To that end, HOMA-IR was calculated as (fasting serum glucose in $\text{mg/dl} \times \text{fasting serum insulin in mU/l})/405$, with lower values indicating a higher degree of insulin sensitivity. The subjects were separated

Table 1
Participant characterization (mean \pm standard deviation).

Parameter	low HOMA-IR	high HOMA-IR
n	19	17
Age	29.51 (4.58)	27.77 (4.51)
BMI	25.23 (2.81)	25.09 (2.50)
HOMA-IR ^a	1.07 (0.23)	2.44 (1.02)
BDI	3.32 (3.54)	3.35 (2.47)
BIS	58.16 (7.35)	59.18 (8.60)

^a Acquired during baseline condition on the testing day, when placebo was applied.

into a low and high HOMA-IR group based on the HOMA-IR values in baseline condition; the threshold was set to 1.5 using a median split. Two participants did have a median HOMA-IR, these were assigned arbitrarily to the lower HOMA-IR group. All participants gave written informed consent to participate in the experiment, which was approved by the local ethics committee of the Medical Faculty of the University of Cologne (Cologne, Germany).

Experimental design. The study was carried out in a single-blinded, placebo-controlled, randomized, crossover design (Fig. 1). Each volunteer participated on four testing days that were 4–21 days apart and started around the same time of the day (either at 8:00 a.m. or 10:00 a.m.). On each testing day, participants arrived fasted with the last meal before 10 p.m. of the previous day.

Each participant received, on different days, either 40, 100 or 160 units of insulin (I.U.; Huminsulin[®] Normal KwikPen 100 I.U./ml; Lilly Germany GmbH) or 1 ml of placebo (Saline KwikPen, Lilly Germany GmbH) in a counterbalanced order. As placebo, the vehicle solution was used that would normally contain the insulin and was, hence, indistinguishable by smell or sensation from insulin. To directly reach the central nervous system and circumvent the blood brain barrier the intervention was administered intranasally. Insulin and placebo were administered with a precision air pump (Aero Pump, Hochheim, Germany) alternating between both nostrils with an interval of 1 min to allow sufficient time for absorption. Each puff of the air pump contained 0.1 ml of solution (equal to 10 I.U.).

On each testing day, prior to subjects' placement in the scanner, an intravenous catheter was inserted into the left forearm vein. The imaging study started with a 10-min task-free fMRI scan (baseline scan). Post-intervention scans were divided into two blocks entailing three 10 min functional runs per block – the first block starting 15 min after the intervention, the second 60 min post-intervention. Subjects were

instructed not to sleep, to lie still and to focus their attention on a dark screen with a central fixation cross. A pulseoxymeter at the left index finger and a flexible pressure belt on their chest were used to record heart rate and respiration rate. Blood samples were drawn before each baseline scan as well as 10, 50, and 90 min post-intervention; glucose, insulin and c-peptide level were measured to control for systemic effects of the intranasally administered insulin. Before the start of each block subjects were asked to rate their hunger, satiety and tiredness on a continuous 100-mm visual analogue scale (0 = “very hungry/sated/tired” and 100 = “not hungry/sated/tired at all”). At the end of each testing day, subjects were further asked to rate whether they received insulin or placebo and to indicate the putative dose on a 100-mm visual analogue scale (0 = low dose, 100 mm = high dose).

Additionally, on the first testing day subjects received a standardized breakfast (one piece of white toast with a slice of cheese or ham) after completing MR-acquisition and blood sampling. To avoid possible hunger-dependent bias the breakfast was provided before the subjects filled in a set of questionnaires comprising the Beck Depression Inventory (BDI-II, Beck et al., 1996) and the Baratt Impulsiveness Scale (BIS-11, Patton et al., 1995).

Imaging parameters. All imaging was performed on a 3T MRI system (Siemens Magnetom Prisma, Erlangen, Germany). Functional imaging data were acquired using a task-free paradigm (“resting state”), lasting 10 min for each of the seven measurements. We used a 64-channel head coil (Siemens, Erlangen, Germany) and 30 axial slices with a T2*-weighted echo-planar imaging sequence (slice thickness: 2 mm; in-plane resolution: 2 mm × 2 mm; no distance factor; ascending interleaved in-plane acquisition; TR = 2000 ms; TE = 30 ms; flip angle = 90°; field of view = 192 × 192 × 60 mm³; PAT factor = 2). After each fMRI block a short anatomical scan was performed first (MPRAGE: 30 slices, voxel size 2 × 2 × 2 mm³, 192 × 192 mm field of view, 250 ms repetition

Within-subject design: dose x intervention x time

Identifying brain response trajectory to insulin on 4 scanning days per participant:

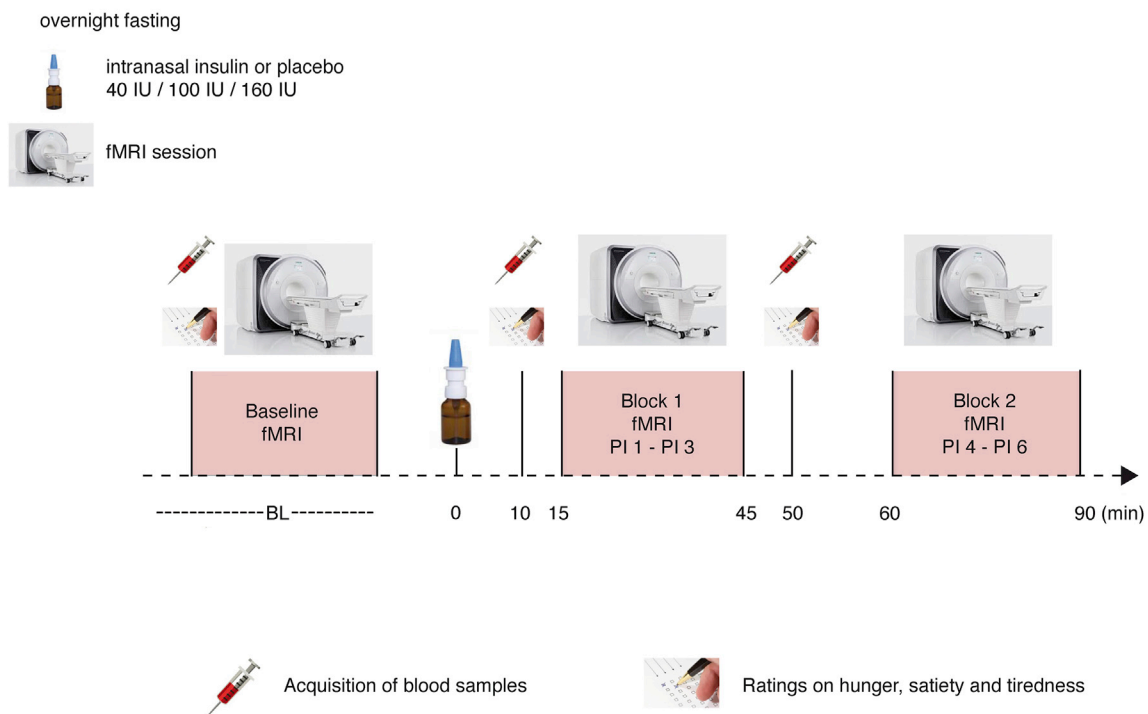


Fig. 1. Placebo-controlled, randomized, crossover design: All volunteers participated on four testing days after an overnight fast. In the baseline condition a blood draw was taken and subjects were asked to rate on hunger, satiety and tiredness before they underwent a 10-min baseline scan. Thereafter, either 40 I.U., 100 I.U. or 160 I.U. of insulin or placebo was intranasally administered in a randomized order. Blood samples and ratings on the internal state were acquired 10, 50, and 90 min post-intervention. We acquired two blocks of task-free fMRI data with three 10-min runs each: The first block covered the interval of 15–45 min, the second one the period of 60–90 min after intervention.

time (TR), 2,86 ms echo time (TE), 0% distance factor), then high-resolution T1-weighted images were acquired using a 12-channel array head coil with 128 sagittal slices that covered the whole brain (MDEFT3D: TR = 1930 ms, TI = 650 ms, TE = 5.8 ms, resolution $1 \times 1 \times 1.25 \text{ mm}^3$, flip angle 18°).

Data analysis. The individual data sets were preprocessed before running statistical analyses using tools from the FMRIB Software Library (FSL version 5.08, www.fmrib.ox.ac.uk/fsl) and according to Smith et al. (2013): Time series were first realigned to correct for small head movements (Jenkinson et al., 2002). Non-brain tissues (e.g., scalp and CSF) were removed using an automated brain extraction tool (Smith, 2002). Data were spatially smoothed using a 6 mm Gaussian FWHM kernel. High-pass temporal filtering was applied (FWHM = 100 s). Structured artefacts were then removed using independent component analysis followed by FSL's ICA-based X-noiseifier (ICA + FIX; Griffanti et al., 2014; Salimi-Khorshidi et al., 2014). Then, functional data were co-registered to the subject's T1-weighted image and normalized to standard space (MNI152, voxel size $1 \times 1 \times 1 \text{ mm}^3$).

Subsequently, statistical data analysis was carried out by using statistical parametric mapping (SPM12; Wellcome Centre for Human Neuroimaging, London) in MATLAB (version 2014b, The MathWorks). This analysis proceeded in two steps: (i) determining subject- and session (time point) -specific functional connectivity of the midbrain; (ii) testing for the effects of time, dose, and either BMI or HOMA-IR on midbrain connectivity.

Subject- and time point-specific functional connectivity. We specified a voxel-wise general linear model (GLM) for each subject comprising intervention dose and time point (resulting in 4×7 separate GLMs per subject). As regressor of interest, these GLMs included the time series of the VTA/SN which was defined anatomically using a mask based on previous magnetization transfer weighted MR images (Bunzeck and Düzel, 2006). Additional regressors of no interest accounted for potential confounds: the six realignment parameters (representing head motion), and two time series from the CSF and white matter, respectively.

The voxel-wise parameter estimates for our regressor of interest represent a linear measure of functional coupling, i.e. the coefficient obtained from regressing the VTA/SN time series on the time series of the respective voxel. In other words, we examined functional connectivity values that reflected the specific effect of insulin over time in the same subject. These subject-, intervention dose- and session-specific contrast images were then entered into a second-level (group-level) GLM.

Group analyses of functional connectivity. To investigate the pharmacokinetics and dose-dependent dynamics of insulin effects on midbrain functional connectivity, first a full-factorial design with the factors dose (4 levels), time point (7 levels, 1 baseline condition and 6 post-intervention time points) and BMI (2 levels) was set up in SPM. This factorial design lends structure to examining the effects of dosage and time while considering the BMI. In a second step, we set up a full-factorial design with the factors dose (4 levels), time point (7 levels) and HOMA-IR (2 levels) to analyse the effect of systemic insulin sensitivity. As results, we only report findings that survived under family-wise-error (FWE) correction at the cluster level ($p < 0.05$), with a cluster-defining threshold (CDT) of $p < 0.001$, across the whole-brain. Notably, under this CDT, cluster-level correction ensures valid false positive rate control (Eklund et al., 2016; Flandin and Friston, 2016). We found a significant cluster reflecting a three-way-interaction for intervention dose, time point and HOMA-IR group in the ventromedial prefrontal cortex (vmPFC). This three-way interaction indicates that an experimental effect exists, specifically, that the dosage-dependent modulation of temporal trajectories of midbrain-vmPFC coupling differ across groups. Since two participants had a median HOMA-IR and were thus assigned arbitrarily to the lower HOMA-IR group, we repeated the analysis after omitting those two subjects and found qualitatively the same three-way interaction. To further disentangle the nature of this complex interaction, we extracted the parameter estimates of this cluster and separately performed two-way ANOVAs for HOMA-IR groups testing the effect of dose

(4 levels) and time-point (7 levels). Next, at each time-point all doses were compared against each other (Fig. 3 a). Multiple comparisons were corrected using Tukey's method. In addition, to visualize insulin dose effects across time beyond the fluctuating placebo effects, we subtracted the parameter estimates in the placebo condition from the corresponding parameter estimates in the insulin condition (Fig. 3b). Given the complexity of our three-way interaction and the many opportunities it affords for testing effects at specific time points, we provide our full data table in the Supplementary Information (Table S3) in order to enable other scientists to analyse the dose-dependent trajectories in both groups according to their specific question.

Other statistical analyses. The analyses of biochemical data and ratings were assessed by linear mixed effect models using the R package 'NLME' 3.1 (Pinheiro et al., 2017). Post-hoc comparisons were calculated using the Tukey's procedure (R 'LSMEANS' package, Lenth, 2016).

3. Results

Neuroimaging data. We examined the modulation of midbrain (VTA/SN) connectivity by intranasal administration of insulin in six time points spread over an interval of 15–90 min post-intervention (Fig. 1).

We did not detect a three-way interaction of interaction dose, time point and BMI (lean and overweight individuals). By contrast, we found a three-way interaction of intervention dose, time point, and peripheral insulin sensitivity (reflected by HOMA-IR) for the functional coupling between the VTA/SN complex and the ventromedial prefrontal cortex (vmPFC; $p = 0.005$, FWE whole-brain cluster-level corrected, under a cluster-defining threshold of $p < 0.001$; see Methods; Fig. 2, Table S2). Two participants did have a median HOMA-IR and were arbitrarily assigned to the lower HOMA-IR group; omitting these subjects from the analysis did not qualitatively affect this three-way interaction (vmPFC, $p = 0.048$, whole-brain cluster-level corrected, under a cluster-defining threshold of $p < 0.001$). In other words, systemic insulin sensitivity rather than body weight interacts with time-dependent modulation of VTA/SN-functional connectivity by intranasal insulin.

The significant three-way interaction indicates that the dosage-dependent modulation of temporal trajectories of midbrain-vmPFC

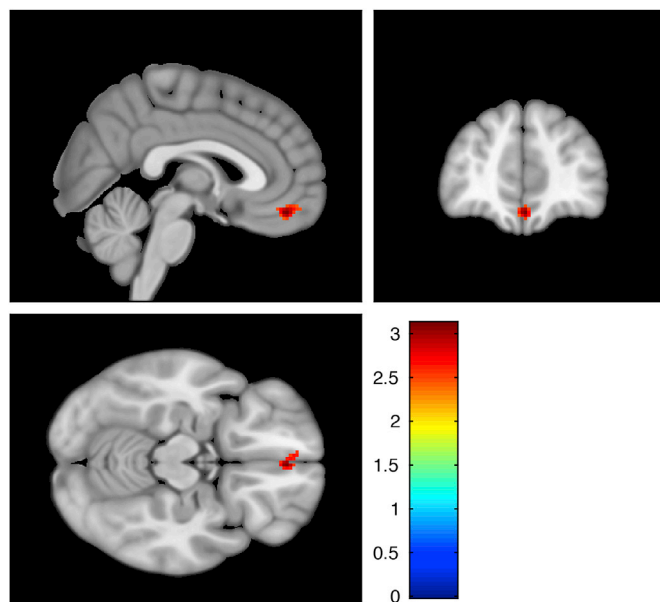


Fig. 2. Modulation of functional connectivity by intranasal insulin. A three-way interaction for dose, time and HOMA-IR revealed coupling between the midbrain (VTA/SN) and the ventromedial prefrontal cortex (vmPFC, $p = 0.005$, whole-brain cluster-level corrected, under a cluster-defining threshold of $p < 0.001$).

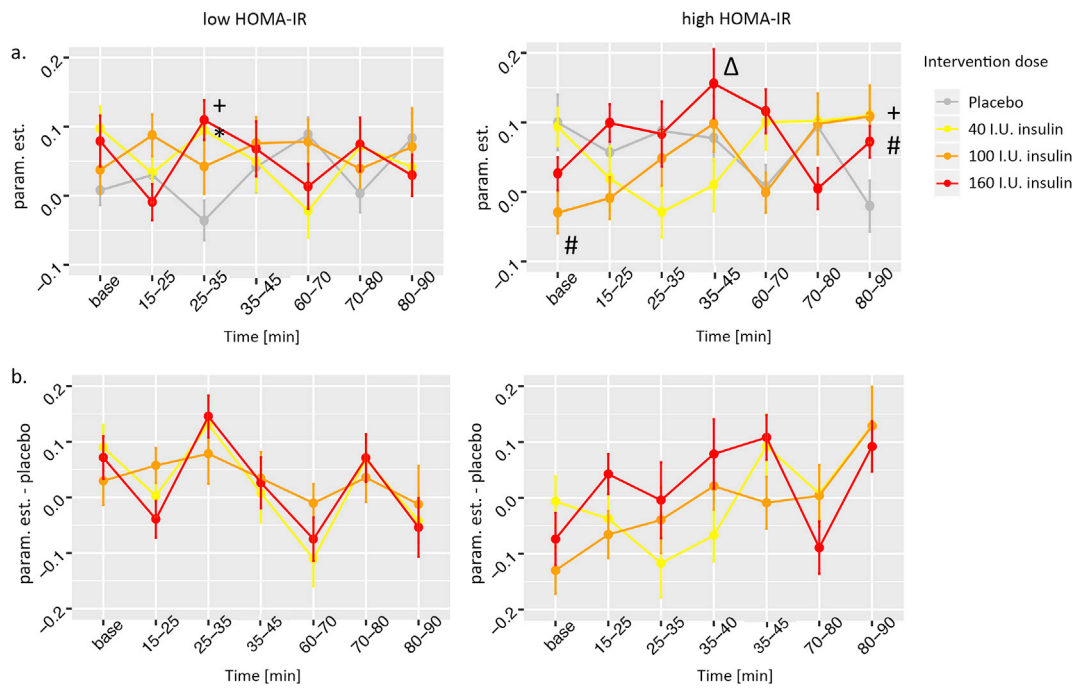


Fig. 3. Dosage effect of intranasally administered insulin on VTA/SN to vmPFC connectivity in subjects with high peripheral insulin sensitivity and subjects with low peripheral insulin sensitivity. a.) The time-by-dose interactions are provided for all doses including placebo. b.) Time-by-dose dynamics of insulin intervention subtracted by parameter estimates in corresponding placebo conditions. x-axis denotes time points (time), y-axis refers to a measure of functional connectivity (parameter estimate from a subject-wise regression model). Data are presented as mean ± SEM; + p < 0.05 for 40 I.U. vs. placebo; # p < 0.05 for 100 I.U. vs. placebo; *p < 0.05 for 160 I.U. vs. placebo, Δp < 0.05 for 160 I.U. vs. 40 I.U. insulin; p-value adjustment for multiple comparisons using Tukey's procedure.

coupling differs across groups. To disentangle this complex result, we analysed the dose-by-time interaction post-hoc, separately for both HOMA-IR groups (Fig. 3). For both the low ($F(18,378) = 1.65, p = 0.046$) and the high HOMA-IR group ($F(18,336) = 2.77, p = 0.0002$) we found a significant dose-by-time interaction.

As illustrated in Fig. 3, the functional coupling between midbrain and vmPFC in the low-HOMA-IR group showed a modulation for 40 I.U. (placebo vs. 40 I.U. insulin, $p = 0.031$) and 160 I.U. (placebo vs. 160 I.U. insulin, $p = 0.012$) of insulin at 25–35 min post-intervention. The high HOMA-IR group exhibited differences already in baseline condition (placebo vs. 100 I.U. insulin $p = 0.047$) and a rather slow increase with significant modulation at 35–45 min (40 I.U. vs. 160 I.U. insulin, $p = 0.019$) and 80–90 min post-intervention (placebo vs. 40 I.U. insulin

$p = 0.046$, placebo vs. 100 I.U. insulin, $p = 0.050$). As explained above, we provide the full data table in the Supplementary Information (Table S3) to enable further exploration of this complex three-way interaction according to specific research questions.

Biochemical data. To ensure adherence to overnight fasting and to control for putative metabolic influences, we checked metabolic parameters and hormones in baseline conditions (i.e., prior to insulin administration): None of these tests showed a significant difference between testing days. High HOMA-IR was associated with higher serum levels of insulin ($F_{1,34} = 24.32, p < 0.001$; Fig. 4b), c-peptide ($F_{1,34} = 13.30, p = 0.0009$; Fig. 4c), as well as insulin-like growth factor 1 (IGF 1; $F_{1,34} = 10.54, p = 0.0026$). Glucose, triglyceride and blood cortisol levels did not differ between HOMA-IR groups.

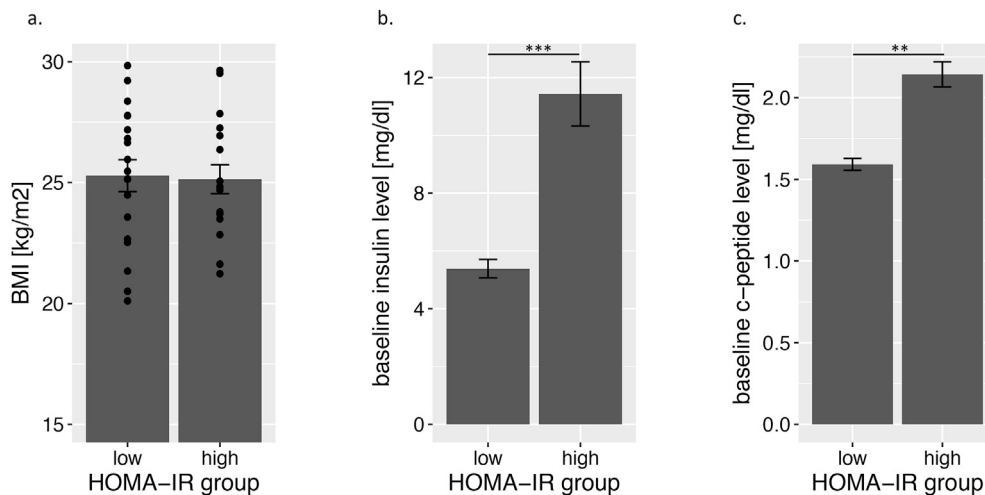


Fig. 4. Differences in peripheral insulin and c peptide levels. a.) HOMA-IR groups showed an equal distribution in BMI but differed in b.) baseline serum insulin level and c.) baseline serum c-peptide level across testing days. Data are presented as means ± SEM; **p < 0.01, ***p < 0.0001.

To assess any potential spill-over of intranasally applied insulin into circulation, we analysed the effect of intranasally administered insulin on metabolic parameters and hormones by collecting blood samples 10, 50, and 90 min, respectively, after intervention. Analysing the effects of intervention dose, time and peripheral insulin sensitivity, we found a time-by-dose (insulin: $F_{9,400} = 19.30$, $p < 0.0001$; c-peptide: $F_{9,398} = 9.78$, $p < 0.0001$), dose-by-HOMA-IR (insulin: $F_{3,102} = 3.27$, $p = 0.0242$; c-peptide: $F_{3,102} = 4.34$, $p = 0.0057$) and time-by-HOMA-IR interaction (insulin: $F_{3,398} = 5.8$, $p = 0.0006$; c-peptide: $F_{3,398} = 7.42$, $p = 0.0001$) for insulin and c-peptide. Ten minutes after intranasal administration of 100 ($t = 6.81$, $p < 0.0001$) and 160 I.U. of insulin ($t = 12.90$, $p < 0.0001$), the serum insulin level increased significantly, suggesting a spill-over effect from the CNS to circulation (Suppl. Fig. S1). The c-peptide level was analysed in addition, given its role in endogenous insulin release: The higher the dose of intranasally applied insulin, the sooner endogenous insulin production decreased. At 160 I.U. the c-peptide level significantly decreased after 10 min ($t = -3.58$, $p = 0.0016$), while at 40 I.U. ($t = -2.60$, $p = 0.011$) and 100 I.U. ($t = -2.99$, $p = 0.005$) the level dropped after 50 min intervention. The change in serum levels of insulin had an impact on the glucose level; the latter showed an interaction of time and dose ($F_{9,402} = 6.59$, $p < 0.0001$). Ten minutes ($t = -4.59$, $p < 0.0001$) and 50 min ($t = -3.83$, $p = 0.0007$) after 160 I.U. of insulin the glucose was significantly decreased (see [Supplementary Material](#) for further details).

Hunger, satiety and tiredness ratings. To control for differences in the internal states between testing days, we instructed the participants to rate hunger, satiety and tiredness immediately prior to the baseline fMRI scan. The ratings were repeated 10, 50 and 90 min after each intervention: We tested the effect of intervention dose, time and HOMA-IR on all three ratings. Hunger ($F_{3,402} = 17.49$, $p < 0.0001$) and tiredness ($F_{3,402} = 25.90$, $p < 0.0001$) increased over time, and satiety rating decreased over time ($F_{3,402} = 18.03$, $p < 0.0001$) but the ratings were not modulated by either insulin dose or HOMA-IR (Suppl. Fig. S2).

4. Discussion

This article provides a systematic analysis of the modulatory effects of intranasally administered insulin on dopaminergic midbrain projections, in two groups of volunteers with different levels of peripheral insulin sensitivity.

The significant three-way interaction detected by our whole-brain analysis suggests that the time- and dose-dependent effects of insulin on the functional connectivity of the midbrain to vmPFC depended on systemic insulin sensitivity. This significantly extends previous findings that the activation of dopaminergic pathways and reward signalling by food cues and actual food intake becomes dysregulated with increasing body weight (de Araujo et al., 2008; Stice et al., 2009, 2010; Wang et al., 2014) and that reward-related and prefrontal neurocircuitry showed reduced insulin responsiveness with increasing BMI (Kullmann et al., 2013). It is important to emphasize, that our results are in line with recent human studies emphasizing that peripheral insulin sensitivity is a better predictor for altered dopaminergic signalling than BMI (Eckstrand et al., 2017; Eisenstein et al., 2015; Tiedemann et al., 2017). This suggests that changes in systemic insulin sensitivity affect midbrain circuits – an interpretation of relevance for the suggestion that insulin (and other humoral factors) provides a nutrient signal that increases incentive salience and motivational drive to obtain food under different metabolic states (Lockie and Andrews, 2013).

In particular midbrain connections to vmPFC are crucial for reward processing and value-based decision making (Jocham et al., 2011). The vmPFC has been associated with encoding value (Rushworth et al., 2012), a construct of central importance for inhibitory control (Appel-hans, 2009; Hare et al., 2009). The values encoded by vmPFC, along with confidence estimates (Lebreton et al., 2015), likely pertain to a large set of actions and stimuli, including the value of health and taste attributes of foods (Medic et al., 2016). Hence, our findings are of interest to recent

suggestions that obesity may be characterized by a shift in how the availability of food influences value-based decision-making (Medic et al., 2016).

To understand the nature of the interaction between time, dose and HOMA-IR, we extracted the parameter estimates from the significant cluster. Interestingly, the lowest (40 I.U.) and highest (160 I.U.) dose of insulin in our study seem to modulate midbrain-vmPFC connectivity with peaks at 30 min after intervention in participants with high systemic insulin sensitivity (Fig. 3). While surprising at first glance, this finding is in line with other human studies reporting central effects of insulin for low as well as for fairly high doses (Suppl. Table S1). Also studies in mice showed a differentiated dose-specific action of insulin on neuronal circuitries with an elevation of reward thresholds by insulin (directly applied in the VTA) for low doses; by contrast, higher doses modulated food intake without affecting reward-related behaviour (Bruijnzeel et al., 2011). We can only speculate about the underlying mechanisms, but it is possible that the effects of insulin on DA receptor function in both VTA and dopaminergic target regions, with consequences for the functional strengths of projections from midbrain, follow a nonlinear (possibly “U-shape”) dose-response relationship, as has been observed for other aspects of insulin function.

The peak at 25–35 min coincided with a previously reported significant change of insulin level in the CSF after intranasal application of the same insulin dose (Born et al., 2002). Born et al. (2002) also reported that after an hour the CSF insulin level had not returned to baseline. Transportation delays after intranasal administration and insulin's multiple effects on DA neurons may also underlie the particular time course we observed (Mittal et al., 2014). Insulin transport to the brain occurs via multiple pathways with different transmission times: (1) intraneuronal transport via the olfactory nerve that takes hours (Born et al., 2002); (2) perineuronal transport along the olfactory and trigeminal nerves within 30 min (Renner et al., 2012); and (3) rapid transport within cerebral perivascular spaces (Lochhead et al., 2015). Once insulin has reached the midbrain, however, it can have various effects on dopaminergic neurons.

Collectively, our findings are thus compatible with the idea that insulin regulates feeding behaviour through its action on midbrain dopamine neurons and the ensuing consequences for reward-related and motivational processes (Davis et al., 2010; Kenny, 2011b). A methodological caveat worth pointing out is that a direct link between functional connectivity of midbrain and changes in dopaminergic signalling is not yet conclusively established in humans. Our approach, which uses functional connectivity as a proxy readout for dopaminergic projections, will thus require further validation.

In conclusion, for the first time we provide an assessment of the time- and dose-dependent effects of insulin on functional connectivity of the dopaminergic midbrain – and suggest that these effects varied with pre-existing difference in insulin sensitivity. Our findings may provide useful guidance for future studies that employ intranasal insulin administration and face the question what insulin dose should be chosen and when functional readouts should be obtained. Furthermore, our results suggest that altered insulin sensitivity impacts on the function of the dopaminergic midbrain and might underlie the dysregulation of reward-related and motivational behaviour in obesity and diabetes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.neuroimage.2019.03.050>.

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