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Dose dependent effects of intranasal insulin on resting-state brain activity

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Context: Insulin action in the human brain influences eating behavior, cognition, and whole-body metabolism. Studies investigating brain insulin rely on intranasal application.
Objective: To investigate effects of three doses of insulin and placebo as nasal sprays on the central and autonomous nervous system and analyze absorption of insulin into the bloodstream.
Design, participants and methods: Nine healthy men received placebo, 40U, 80U and 160U insulin spray in randomized order. Before and after spray, brain activity was assessed by functional magnetic resonance imaging and heart rate variability (HRV) was assessed from ECG. Plasma insulin, C-peptide, and glucose were measured regularly.
Setting: general community
Results: Nasal insulin administration dose-dependently modulated regional brain activity and the normalized high-frequency component of the HRV. Post-hoc analyses revealed that only 160U insulin showed a significant difference from placebo. Dose-dependent spill-over of nasal insulin into the bloodstream was detected. The brain response was not correlated with this temporary rise in circulating insulin.
Conclusions: Nasal insulin dose-dependently modulated regional brain activity with the strongest effects after 160 U. However, this dose was accompanied by a transient increase in circulating insulin concentrations due to a spillover into circulation. Our current results may serve as a basis for future studies with nasal insulin to untangle brain insulin effects in health and disease.

We investigated the effect of insulin on the central and autonomous nervous system. We detected dose-dependent effects of intranasal insulin on regional brain activity and parasympathetic tone. .

Introduction

Research over the last years identified the human brain as an insulin sensitive organ (1, 2). In response to the hormone, the central nervous system regulates various functions as the response to food cues, reward processes, and memory (1). Furthermore, insulin effects in the brain impact the rest of the body modulating peripheral insulin sensitivity, thermogenesis as well as liver and lipid metabolism (2, 3). However, a substantial number of individuals are brain insulin resistant and therefore lack these effects of the peptide. The best characterized factor associated with brain insulin resistance is body weight with reduced insulin effects in overweight and obese persons. Beyond this, a number of other factors have been identified thus far (1, 2). However, it is still not known whether factors associated with brain insulin resistance represent cause or consequence thereof.

To assess brain specific insulin effects in humans, most research applied insulin as a nasal spray (2). Research in animals demonstrated that peptide transporters near the olfactory bulb rapidly transport a limited number of peptides (including insulin) into the cerebrospinal fluid (CSF) from where they further reach brain cells (4). In humans, a rise in CSF insulin concentrations was detected as early as 10 minutes after administration of 40U of the peptide as nasal spray (5). Functional consequences, e.g. effects on brain activity occur in a comparable time frame (6).

The earliest experiments mostly used a dose of 160 U to investigate the consequences of intranasal insulin application on eating behavior and body weight; studies investigating memory function in cognitively impaired patients, on the other hand, used a lower dose of insulin (7, 8). An acute dose of 160 U has been shown to stimulate regional brain activity in a number of brain areas (9). While initial experiments with a lower insulin dose suggested that none of the peptide reach the circulation after administration as a nasal spray (5), it recently became evident that small amounts of human insulin are indeed absorbed into the bloodstream and are detectable in venous blood (10-12). After application of 160 U of insulin as a nasal spray, around 0.1 U enter the blood stream (12, 13). Despite having no major effects on blood glucose (e.g. not inducing hypoglycemia), the small rise in circulating insulin may induce effects in peripheral tissues, possibly influencing the interpretation of metabolic effects of nasal insulin.

Furthermore, experiments with chronic administration of human insulin as nasal spray suggested that high doses might not necessarily have the strongest effects. Indeed, in a four months trial in cognitive impaired persons the lower dose of 20 U of the peptide had more profound effects on memory than 40 U (14).

Even though the intranasal administration has already been used in a number of studies in humans (6, 7, 15-21), there is still no systematic comparison of the acute effect of different nasal insulin doses on central and autonomous nervous system activity. We therefore investigated the effects of placebo and three different doses of human insulin as nasal spray. Before and after spray, brain activity was assessed by functional magnetic resonance imaging while simultaneously recording electrocardiograms (ECG) to assess heart rate variability (HRV). Frequent blood sampling was used to analyze the absorption of insulin into the bloodstream.

Material and Methods

Participants

Nine healthy men participated in the study (BMI 20-26kg/m^2, age 23-30 years) (Table 1). Informed written consent was obtained from all subjects and the local Ethics Committee approved the protocol. To ensure that participants were healthy and did not suffer from

psychiatric, neurological nor metabolic diseases, they underwent a thorough medical examination.

Study design

Volunteers received placebo, 40U, 80U and 160U insulin as nasal spray in randomized order (on four separate days with 7-14 days time-lag) with repetitive MRI and ECG measurements (see Figure 1). Participants were blinded to the condition. Experiments were conducted after an overnight fast and started at 7.00 a.m. with a baseline MRI measurement including two resting-state BOLD fMRI and a cerebral blood flow measurement (**rsfMRI1** and **CBF1**). After the basal measurement, the respective nasal spray was administered. After 15 and 30 minutes, a second and third MRI measurement was performed (**rsfMRI2/CBF2** and **rsfMRI3/CBF3**). During all three MRI measurements ECG was recorded. The subjective feeling of hunger was rated at two time points (before spray application 60 min after intranasal spray) on a visual analogue scale from 0 to 10 (0: not hungry at all; 10: very hungry).

Participants rated 100 food pictures in two separate blocks according to explicit "liking" (how much do you like the food item in general) and "wanting" (how much would you like to eat the food item right now) on each study day.

Application intranasal insulin/placebo

On each day, participants received 1.6 ml of nasal spray. It contained either placebo, 40, 80, or 160 U of human insulin (Insulin Actrapid; Novo Nordisk, Bagsvaerd, Denmark). Under supervision, the spray was administered over four minutes with two puffs per nostril every minute.

Blood measurements

Venous blood samples were obtained immediately before as well as 5, 10, 15, 20, 30, 60, 90, and 120 minutes after spray application.

Plasma glucose concentrations were measured using the glucose-oxidase method (Yellow Springs Instruments, Yellow Springs, USA). Serum insulin, C-peptide, cortisol, luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, and thyroid-stimulating hormone (TSH) were measured by chemiluminescence assays on the ADVIA Centaur XPT and Adrenocorticotropic hormone (ACTH) by an automated solid-phase, chemiluminescent immunoassay on the Immulite XPT analyzer (both Siemens Healthineers, Eschborn, Germany).

Whole-brain fMRI measurement

Data acquisition

Scanning was conducted at a 3T whole-body Siemens scanner (Magnetom Prisma; Erlangen, Germany) with a 20-channel coil. Three different types of functional data sets were recorded each day before and after nasal spray application. In addition, high-resolution T1 weighted anatomical images were obtained.

To acquire CBF maps, pseudo-continuous arterial spin labeling (PCASL) was performed using a 2D EPI read out sequence (22) with background suppression. A total of 52 images were acquired with the following parameters: 16 slices, slice thickness 3mm, 1.5mm gap, TR=4500ms, TE 23ms, FOV_{read}=192mm², FOV_{phase}=100%, matrix 64x64, flip angle 90°, voxel size 3x3x3 mm^3, bandwith 2004px/Hz, tag gradient strength = 7.0mT/m. The first image volume prior to the preparation scans was used for calibration (M_0).

Whole-brain BOLD data were collected by using echo-planar imaging sequences, as recently reported (21).

To acquire BOLD activation of subcortical regions at a higher resolution, an echo-planar imaging sequence with ZOOMit was used. It uses dynamic excitation pulses to achieve selective field-of-view (zoomed) images, without aliasing artifacts. The following parameters were used: TR=3s, TE=34ms, FOV_{read}=192 mm², FOV_{phase}=33.3%, matrix 96x32x 36, flip angle 90°, voxel size 2x2x2.5mm^3, slice thickness 2mm, images were acquired in ascending order. Each brain volume comprised 36 axial slices and each functional run contained 60 image volumes, resulting in a total scan time of 3:06 minutes.

Before intranasal insulin application and after 30 minutes all fMRI measurements were performed as displayed in Fig. 1. Due to time constraints only CBF and BOLD of subcortical regions was performed after 15 minutes.

PCASL preprocessing:

Preprocessing was performed using FSL with the following tools (5.0.9). First, the images were realigned using *mcflirt*. The resulting 4D ASL data were processed using o*xford_asl*. For CBF quantification a single compartment standard kinetic model was used (23). Perfusion images (ΔM) were obtained by pairwise subtraction of tag and control images. A voxel wise calibration was performed thereby correcting possible RF coil inhomogeneity (23). In a next step, the T1-image was coregistered with the M0-image and the resulting transformation parameters were applied to the CBF maps. Finally, the CBF maps were normalized in MNI space using SPM12.

Resting-state fMRI data preprocessing

We used the Data Processing Assistant for Resting-State fMRI (24) to analyze the resting state fMRI data, which is based on Statistical Parametric Mapping (SPM12) and Resting-State fMRI Data Analysis Toolkit (25) (REST, http://www.restfmri.net). The whole-brain functional images were normalized to voxel size: 3x3x3 mm^3 and the subcortical functional images to 2x2x2 mm^3 and then smoothed (FWHM: 6mm for whole-brain and 4mm for the subcortical images). Nuisance regression was performed using white matter, CSF, and the six head motion parameters as covariates.

To investigate resting-state brain activity, we calculated the fractional amplitude of low-frequency (0.01-0.08 Hz) fluctuations (fALFF) of the blood oxygenation level dependent (BOLD) signal (26). The regional intensity of spontaneous BOLD fluctuations is quantified by the power spectrum in the low frequency range (0.009-0.08 Hz) and regularized by the power in the whole frequency range (0-0.25 Hz) (26).

Statistical analyses

Cerebral blood flow

CBF values were extracted of the hypothalamic region of interest based on recent findings. Here, we identified the hypothalamus to respond with a persistent decrease in activity up to 30 min after intranasal insulin application (6, 10, 19, 20). This response was diminished in individuals with unfavorable fat distribution and whole-body insulin resistance.

Baseline-corrected CBF maps were computed to quantify the hypothalamic CBF change 15 and 30min after intranasal insulin application (CBF2 minus CBF1 and CBF3 minus CBF1, respectively). Repeated measurement ANOVA (factor insulin dose with four levels: Placebo, 40U, 80U and 160U Insulin; and factor *time* with two levels) with HOMA-IR as covariate was performed in SPSS (IBM, version 20) ($p<0.05$).

Resting-state fMRI

fALFF maps of the subcortical regions were baseline corrected (rsfMRI2 minus rsfMRI1; rsfMRI3 minus rsfMRI1). Repeated measurement ANOVA (full factorial model) was performed

in SPM12. Significant clusters were extracted for post hoc analyses in SPSS (IBM; version 20). A statistical threshold of $p_{FWE}<0.05$ voxel-level whole-brain corrected was applied. Additionally, small volume correction was performed for the hypothalamus, *a priori* region of interest (6, 10).

All whole-brain fALFF maps were baseline corrected (rs*fMRI3* minus *fMRI1*). Repeated measurement ANOVA/ full factorial model was performed in SPM12. Significant clusters were extracted for post hoc analyses in SPSS (IBM; version 20). A statistical threshold of $p_{FWE}<0.05$ voxel-level whole-brain corrected was applied. Additionally, small volume correction was performed for the prefrontal cortex (PFC), *a priori* region of interest, based on recent findings showing that activity and functional connectivity can be enhanced with intranasal insulin (6, 19, 21) .

Heart rate variability

Electrocardiograms were recorded with BIOPAC MP35 (BIOPAC, Goleta, CA) and a sampling rate of 1,000 Hz, during the whole fMRI recording. For analysis, we extracted 5 minutes of recording during the CBF measurements. The data were analyzed with kubios (http://kubios.uef.fi). HRV parameters were calculated on individual R-R interval time series in the low-frequency (0.04–0.15 Hz) and high-frequency (0.15–0.40 Hz) bands. HRV parameters were baseline corrected and analyzed using a repeated measurement ANOVA in SPSS (IBM, version 20) ($p<0.05$).

Blood values

The areas under the curve (AUC) were calculated using the trapezoid rule. Doses were compared by linear regression analyses with dose as a continuous variable ($p<0.05$).

Results

Dose dependent effects of intranasal insulin on brain activity

Hypothalamic cerebral blood flow (CBF) change

Based on our recent finding, we assessed dose-dependent insulin induced hypothalamic change in CBF as readout for hypothalamic insulin sensitivity (6, 10, 12, 27). We identified a significant main effect of dose ($F(3)=5.36$, $p=0.01$) (Figure 2) and a significant interaction between dose and peripheral insulin sensitivity assessed by HOMA-IR ($F(3)=4.43$, $p=0.02$). No main effect of time (15 versus 30 min post spray) was observed. Post-hoc analyses revealed a significant CBF difference between placebo and 160 U of intranasal insulin ($p=0.04$, Bonferroni corrected). Furthermore, a significant positive correlation was observed between HOMA-IR and the hypothalamic response to 160 U of insulin 30 min after application ($r=0.76$, $p=0.017$).

Subcortical resting-state fMRI response using fALFF

We observed a significant main effect of dose in the left amygdala ($p_{FWE}=0.05$, Figure 3A, table 1). A significant linear decrease with insulin dose was observed in the right caudate nucleus ($p_{FWE}=0.04$, Figure 3B, table 1) and the hypothalamus ($p_{FWE}=0.015$, small volume corrected, Figure 3C, table 1). Since no main effect of time or interaction between time and condition were observed ($p_{FWE}>0.05$), post-hoc analyses were performed on mean response 15 and 30 min after nasal spray. Post hoc analyses showed a significant fALFF difference in the hypothalamus between placebo and 160U insulin sprays ($p=0.003$) and between placebo and 80U ($p=0.014$). For the left amygdala, we observed a significant difference between placebo and 160U ($p<0.001$), between 40U and 160U ($p<0.001$), between 80U and 160U ($p=0.004$) and a statistical trend for difference between placebo and 80 U ($p=0.07$). For the caudate response, a significant

difference between placebo and 160 U (p=0.003) and between 40 U and 160 U (p=0.003). No interactions were observed with peripheral insulin sensitivity assessed as HOMA-IR.

Whole-brain resting-state fMRI using fALFF

We observed a significant linear increase in the lateral prefrontal cortex (p_{FWE}=0.02 small volume corrected, Figure 3D, table 1) with increasing insulin doses. Post hoc analyses showed a significant difference between placebo and 160 U (p<0.001), placebo and 80 U (p=0.015), between 40 U and 160 U (p=0.001) and between 40U and 80U (p=0.045). No interactions were observed with HOMA-IR.

Whole-brain and subcortical resting-state fMRI results are summarized in table 2.

All post-hoc results were Bonferroni corrected.

Behavioral results

We acquired subjective feeling of hunger on a visual analogue scale before and 60 min after nasal spray. We observed no acute effect of insulin dose on hunger and no interaction between insulin dose and time (p>0.05). There was a significant effect of time on hunger (F(1)=1.64; p=0.009).

Furthermore, subjects rated liking and wanting for high caloric sweet and savory foods 60 min after nasal spray. No main effect of dose was observed. However, exploratory correlation analysis revealed a significant positive association between the caudate response to 160 U insulin and liking for sweet foods (r=0.763; p=0.008).

Heart rate variability

To assess possible dose dependent effects on the autonomous nervous system, we recorded ECG during each MRI measurement. We detected a slight decrease in absolute heart rate after any of the tested nasal sprays including placebo (Maine effect of time: F(2)=6.4; p=0.016). Though, there was a significant effect of insulin dose on the normalized high frequency band (F(3)=3.56; p=0.047) and a significant insulin dose by time interaction (F(3)=10.52; p=0.001). Post hoc analyses revealed a significant difference between placebo and 160U response (p=0.026) and between time point 15 and 30 min (p=0.03). On the normalized low frequency band, we observed a statistical trend for main effect of dose (F(3)=3.30; p=0.058). Post hoc analysis revealed no significant differences between conditions. To explore brain-peripheral interactions we correlated the change of the high frequency band with the brain response to placebo and insulin as recently reported (10). The hypothalamic CBF response correlated positively with the high-frequency change after 160 U of insulin adjusted for BMI (r_{adj}=0.940; p=0.018). No such correlation was observed after any other insulin dose.

Blood data

After insulin spray administration, there was a dose-dependent increase in circulating insulin concentrations ($p_{AUC\ 0\text{-}30min}$=0.01) of 6.9±19.3 pmol/l after 40U, 17.2±11.8 pmol/l after 80U, and 30.9±29.8 pmol/l after 160U while insulin levels slightly decreased and non-significantly after placebo spray by 20.9±23.7 pmol/l. Insulin concentrations reached their peak at 15 minutes post insulin spray and returned to pre-spray levels another 15 minutes later (Figure 4 A and B).

No correlations between increase in plasma insulin (quantified as both incremental AUC 0-30 minutes and peak increase) and any of the brain responses described above was detected (p>0.05).

Neither serum C-peptide concentrations nor plasma glucose levels were different between the days ($p_{AUC\ 0\text{-}120}$=0.9 and $p_{AUC\ 0\text{-}120}$=0.6, respectively; Figure 4 C and D).

Furthermore, no differences between days were detected for LH, FSH, and testosterone, for ACTH and cortisol, and for TSH concentrations (all $p_{AUC\ 0\text{-}120} \geq 0.6$, supplementary Figures 1 and 2).

Discussion

In the current study, we investigated the effect of different insulin doses applied intranasally on the central and autonomous nervous system as well as peripheral metabolism. We detected dose-dependent effects of intranasal insulin on brain activity and regional blood flow. The hypothalamus, amygdala, caudate nucleus, and the lateral prefrontal cortex showed a most prominent effect for 160 U human insulin compared to placebo. Furthermore, we identified a dose-dependent increase in circulating insulin concentrations as well as an increase in high frequency band activity of the autonomous nervous system.

We were able to replicate previous findings on insulin action in specific human brain areas (9). However, high doses of insulin nasal spray (e.g. at least 80 U) were necessary to acutely introduce detectable changes in brain activity in these regions. While much lower insulin doses as 20 U may be beneficial to study chronic effects on complex brain functions (14), acute quantification of regional insulin effects by fMRI seems to require higher doses e.g. the frequently applied 160 U. As we did not detect significant effects of 20 and 40 U of nasal insulin, our results raise the question of the underlying mechanisms of such chronic effects of low dose nasal insulin. These may include repeated sub-threshold activations of specific regions, but could also be related to changes in the milieu of the brain that might arise under chronic exposure.

When addressing effects of nasal insulin on metabolic function, higher insulin doses have a potential disadvantage by temporarily increasing circulating insulin. We now precisely quantified this for the first time in a dose-dependent manner. As the excursion of insulin is not accompanied by changes in C-peptide, which would indicate endogenous origin, the rise in insulin can only be caused by a spillover of spray into the bloodstream. This spillover has been detected before after 160 U of nasal insulin and corresponds to an intravenous insulin dose of approximately 2.5 mU / kg body weight (12) or an absolute dose of 0.1 mU (13) for this nasal insulin dose. Furthermore, the spillover seems to be different between human insulin, as used in our current study, and rapid-acting insulin analogues. While application of 40 U human insulin caused only minor changes in plasma insulin, 40 U of the rapid-acting insulin lispro introduced marked rises in circulating levels in a recent study (28). Furthermore, the kinetics of intranasal human insulin and the insulin analog lispro seem to be quite different. A delayed absorption of insulin lispro reached peak levels in the blood at least 15 minutes later than human insulin (28). By this time, plasma insulin levels were already back to baseline after human insulin spray.

The magnitude of the insulin spillover into circulation was not related to any of the detected brain effects in our study. Hence, penetration of the peptide directly into the brain and not the transport via the blood stream seems to be the major mode of action of nasal insulin in the CNS. While not routinely done in the past (15, 20, 29), our current results on insulin spillover indicate that this phenomenon should be mimicked by intravenous insulin application when studying peripheral tissues. Of note, we did so in one recent experiment: In this study, insulin delivery to the brain via insulin nasal spray improved peripheral insulin sensitivity by suppressing endogenous glucose production and stimulating glucose uptake into peripheral tissues independent of insulin spill over (12).

The insulin-induced change in heart rate variability substantiates previous results on the role of the autonomous nervous system (20). Intranasal insulin specifically induced change in the

high frequency band, which represents mainly the parasympathetic branch of autonomous nervous system. This is well in line with animal data showing that central insulin action is transmitted via the major parasympathetic nerve, i.e. the vagus nerve, to peripheral organs (30). Just as in animals, the hypothalamus seems to contribute to this response, as it plays a pivotal role for homeostatic regulation and integrating metabolic signals. Insulin reactivity of the hypothalamus has been shown to be compromised in obese individuals (6, 21, 27). Previous results in humans indicated that parasympathetic outflows from the hypothalamus contribute to the modulation of peripheral insulin sensitivity (20). For other aspects of peripheral metabolism (e.g. lipolysis or liver metabolism) this has not been investigated yet. Our current results indicate that future studies aiming to monitor the autonomous nervous system should apply higher doses of insulin nasal spray, as we only detected significant effects after 160 U.

Another possibility of brain-derived modulation of peripheral metabolism are endocrine signals. Potential mechanisms involve the hypothalamic–pituitary–adrenal (HPA) axis, the hypothalamic–pituitary–gonadal axis, or modulation of thyroid function. Just as previously reported in a larger group for cortisol (10), we detected no effects of nasal insulin on the HPA axis. Furthermore, the pituitary-gonadal and thyroid axes were unaffected by nasal insulin. However, we cannot exclude that chronic nasal insulin administration may have effect on endocrine functions (especially gonadal), as brain-specific knockout of the insulin receptor impaired LH regulation and thereby reproductive function (31) in rodents. As we did not detect acute effects of nasal insulin on the assessed endocrine functions, it is most likely that the autonomous nervous system is the key transducer for acute peripheral effects.

Besides the hypothalamus, a dose-dependent effect of insulin was found in regions recently identified as insulin sensitive with strong relevance for food intake and body weight regulation. The amygdala and lateral PFC showed a significant increase for 160 U of insulin, while the caudate nucleus (striatal region) revealed a significant decrease in resting-state activity.

The hypothalamus plays a vital role in whole-body energy homeostasis. In animal studies, insulin signaling is similar in the amygdala and the hypothalamus including obesity related dysregulation (32). Furthermore, both are embedded in the striato-prefrontal circuitry (33, 34), which is particular sensitive to increasing peripheral and central insulin levels (for a recent review see (1, 9, 21). Furthermore, as seen in the current study, intranasal insulin influences neural activity in the striatum and prefrontal cortex (6, 10, 20, 35-37). Moreover, animal models have shown multiple interactions between homeostatic and reward brain circuits (38) in particular that insulin can amplify dopamine release in the striatum (39). Hence, the marked insulin-induced hypothalamic decrease can potentially increase satiety, while the decrease in caudate activity and increase in amygdala and PFC activity to insulin may attenuate the rewarding properties of food and motivation for food consumption.

Taken together, we detected dose-dependent effects of intranasal insulin on regional brain activity and parasympathetic tone. While no acute effects of 40 U of nasal insulin were observed, 160 U of the peptide had the strongest effects. However, this dose was accompanied by a transient increase in circulating insulin concentrations due to a spillover into circulation. This is no major obstacle for the assessment of regional brain effects by imaging but should be mimicked in studies on peripheral metabolism. Taking this into account, intranasal insulin application is currently the best available tool to dissect central from peripheral insulin effects. Our current results can be the basis for the design of future studies with nasal insulin administration to disentangle brain insulin effects in health and disease.

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Figure 1. Schematic overview of study design. Cerebral blood flow (CBF), resting-state fMRI (rsfMRI) for subcortical regions and whole-brain were acquired at different time points before and after intranasal placebo and insulin sprays. ECG was recorded during the entire MRI acquisition. Blood samples, indicated by red symbols, were taken before, 5, 10, 15, 30, 60, 90, and 120 min after intranasal placebo and insulin sprays.

Figure 2. Dose dependent intranasal insulin effect on hypothalamic cerebral blood flow (CBF). Bar plot shows the extracted CBF values adjusted for HOMA-IR from before to after nasal spray application of placebo, 40U, 80U, and 160U insulin. Post-hoc analyses showed significant differences between placebo and 160U of insulin (*p<0.05-Bonferroni corrected).

Figure 3. Dose dependent intranasal insulin effect on resting-state fMRI response. Bar plots show the extracted mean z-values of significant fALFF clusters from before to after nasal spray application of placebo, 40U, 80U and 160U insulin. Post-hoc analyses showed significant differences between conditions as indicated by asterisks (*p<0.05-Bonferroni corrected; **p<0.005-Bonferroni corrected). **(A)** Main effect of insulin dose in the left amygdala displayed by yellow-color coded *F*-value map (p<0.05, FWE-corrected). Since no main effect of time or interaction between time and condition were observed, post-hoc analyses were performed on mean response 15 and 30 min after nasal spray. **(B)** Linear decrease of insulin dose in the right caudate displayed by yellow-color coded *T*-value map (p<0.05, FWE-corrected). Since no main effect of time or interaction between time and condition were observed, post-hoc analyses were performed on mean response 15 and 30 min after nasal spray. **(C)** Linear decrease of insulin dose in the hypothalamus displayed by yellow-color coded *T*-value map (p_{svc}<0.05, FWE-corrected). Since no main effect of time or interaction between time and condition were observed, post-hoc analyses were performed on mean response 15 and 30 min after nasal spray. **(D)** Linear increase of insulin dose in the prefrontal cortex displayed by yellow-color coded *T*-value map (p_{svc}<0.05, FWE-corrected). Bar plot shows the extracted mean z-values of prefrontal cortex fALFF from before to 30 min after nasal spray application for the different conditions.

Figure 4 – Effect of intranasal insulin on peripheral metabolism. At 0 minutes, nasal spray was administered. Filled circles represent measurements after administration of placebo spray, open circles after application of 40 U of nasal insulin, filled triangle after 80 U intranasal insulin, and open triangles after 160 U of insulin spray. (A) shows serum insulin concentrations, (B) presents the absolute change in plasma insulin concentration from baseline (0 minutes). (C) shows plasma glucose levels, and (D) serum C-peptide concentrations. Presented are means ± SEM. Differences between insulin spray concentrations were adressed by comparisons of areas under the curves (AUC) for the indicated time intervals using insulin dose as a continuous variable.

Table 1. Characteristics of nine male participants

Variable	Mean ± SD (Range)
Age (years)	26.56 ± 2.78 (23-30)
Body Mass index (kg/m^2)	23.44 ± 2.01 (20.09-26.01)
Body fat content (%)	18.9 ± 2.4 (16.6-21.9)
Waist-to-hip ratio	0.91 ± 0.07 (0.81-0.99)
HbA1c (%)	5.2 ± 0.1 (5.1-5.4)
HOMA-IR (mean overall 4 measurement days)	2.25 ± 0.41 (1.51-2.91)

Table 2. Resting-state fMRI response to different concentrations of insulin

Brain regions	MNI coordinate (x,y,z)	P value *FWE*-corr	z value	Post-hoc analyses (p<0.05, Bonferroni corrected)
Main effect of insulin dose (F contrast)				
Amygdala	-20, 2, -18	0.05	4.87	PBO<160U; 40U<160U; 80U<160U
Linear decrease to insulin dose (T contrast)				
Caudate	18, 6, 18	0.04	4.91	PBO>160U; 40U>160U
Hypothalamus	-8, -2, -8	0.01*	3.58	PBO>80U; P>160U
Linear increase to insulin dose (T contrast)				
Superior frontal gyrus	18, 39, 45	0.02*	3.92	PBO<80U; P<160U; 40U<80U; 40U<160U

Data from full factorial model investigating the effect of insulin dose (Placebo, PBO; 40U, 80U and 160U of insulin). A statistical threshold of p<0.05, family-wise error (FWE) correction was used, *small volume corrected.