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ORIGINAL ARTICLE

Intranasal insulin administration decreases cerebral blood flow in cortico-limbic regions: A neuropharmacological imaging study in normal and overweight males

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Abstract

Aim: To assess and compare the effects of 160 IU intranasal insulin (IN-INS) administration on regional cerebral blood flow (rCBF) in healthy male individuals with normal weight and overweight phenotypes.

Methods: Thirty young male participants (mean age 25.9 years) were recruited and stratified into two cohorts based on body mass index: normal weight (18.5-24.9 kg/m^2) and overweight (25.0-29.9 kg/m^2). On separate mornings participants received 160 IU of IN-INS using an intranasal protocol and intranasal placebo as part of a double-blind crossover design. Thirty minutes following administration rCBF data were collected using a magnetic resonance imaging method called pseudocontinuous arterial spin labelling. Blood samples were collected to assess insulin sensitivity and changes over time in peripheral glucose, insulin and C-peptide.

Results: Insulin sensitivity did not significantly differ between groups. Compared with placebo, IN-INS administration reduced rCBF in parts of the hippocampus, insula, putamen, parahippocampal gyrus and fusiform gyrus in the overweight group. No effect was seen in the normal weight group. Insula rCBF was greater in the overweight group versus normal weight only under placebo conditions. Peripheral glucose and insulin levels were not affected by IN-INS. C-peptide levels in the normal weight group decreased significantly over time following IN-INS administration but not placebo.

Conclusion: Insulin-induced changes within key regions of the brain involved in gustation, memory and reward were observed in overweight healthy male individuals. Following placebo administration, differences in gustatory rCBF were observed between overweight and normal weight healthy individuals.

KEYWORDS

cerebral blood flow, hippocampus, intranasal insulin, normal weight, overweight

1 | INTRODUCTION

Obesity has been classified as a major public health issue and its prevalence continues to increase, with more than approximately two-thirds of adults in the UK currently living with obesity and overweight (OW).[1] Coupled with this is the increase in type 2 diabetes (T2D), a disease associated and largely defined through an insensitivity to the peripheral effects of insulin, termed insulin resistance.[2] Insulin effects in the peripheral system have been well evaluated and researched, however, insulin's effects on brain function have yet to be fully elucidated. A body of behavioural and neuroimaging literature has shown a modulatory role of insulin in mechanisms linked to appetite control and food intake.[3] Some of these studies have delivered human insulin solution via the nasal cavity, a procedure commonly termed intranasal insulin (IN-INS) administration. This method permits direct brain administration, circumventing peripheral blood glucose regulation and control,[4] increasing cerebrospinal fluid (CSF) levels within 30-60 minutes.[5] In comparison with intravenous and oral methods, IN-INS is an attractive tool for assessing the effects of insulin on brain function because of its limited effects on peripheral glucose concentration and other metabolic gut-derived hormones.[6,7] Previous functional neuroimaging research with IN-INS has shown little direct interaction with cerebral vasculature,[7] suggesting that insulin-associated effects seen from neuroimaging techniques, which take advantage of neurovascular coupling, are a product of neuronal insulin signalling.[8] Given this, functional MRI (fMRI) methods in combination with intranasal delivery offer a valuable way to investigate the effects of insulin on brain function.

OW is a term that describes an individual with a body mass index (BMI) above the range considered healthy or desirable. The OW phenotype is defined as a BMI range of 25-29.9 kg/m^2 and in medicine is considered a precursor to obesity (a BMI of 30 kg/m^2 or higher). Obesity is associated with many morbidities, including T2D and increased cardiovascular risk.[9] Importantly, population studies have shown increased T2D diagnoses,[10] cardiovascular-related death[11] and overall mortality[12] with incremental BMI increases above 25 kg/m^2, classifying those who are OW (but not obese) as an at-risk population for the aforementioned morbidities. In England, ~40% of adult males are OW as assessed by BMI, with ~33% of individuals classified as normal weight and ~26% as obese, therefore the majority of males in England are classified as OW.[1]

There is considerable interest in the impact of IN-INS in those individuals with impaired appetite control. Reports show a lack of effective weight loss following long-term IN-INS administration in men with obesity compared with men of normal weight who achieved a significant weight loss.[13] To our knowledge, this is the first study of the impact of IN-INS on people in this intermediate non-obese category exclusively. Studying this group of individuals is important for establishing possible markers and differences in brain function and/or central insulin sensitivities that may be associated with this healthy, but potentially at-risk population.

A small number of experiments looking at the modulatory effects of IN-INS have employed arterial spin labelling (ASL) to explore regional cerebral blood flow (rCBF) changes in normal weight,[6] obese, insulin-resistant[14] and also elderly individuals.[15] Measures of rCBF with ASL are quantitative (ml/100 g of brain tissue/minute) and seemingly physiologically relevant when examining drug-induced effects in the brain.[16]

The present study is, to our knowledge, the first to measure rCBF in response to IN-INS versus IN-placebo (IN-PLA) in a group of healthy normal weight and OW individuals as determined by BMI. Based on the previous literature, using this dose, we hypothesized that 160 IU IN-INS would produce significant changes in rCBF within the limbic and cortical structures that express insulin receptors.[17] We further predicted that OW individuals may display decreased central insulin sensitivity and this would be observed as less pronounced changes in rCBF in contrast to normal weight comparators.

2 | MATERIALS AND METHODS

2.1 | Participants

The study followed the guidelines in the Declaration of Helsinki and was approved by the King's College London Psychiatry Nursing and Midwifery Ethics Committee (RESCM-17/18-2282). Written, informed consent was signed prior to any study procedures. The study comprised three visits. The first was a screening session and the remaining two visits were experimental imaging sessions. The two imaging sessions were separated by ~1 week.

Healthy right-handed male participants were screened to ensure they had no history of psychiatric illness or diabetes, no cardiac-related complications, no history of any eating disorders, asthma or allergies associated with breathing difficulties, and did not smoke more than five cigarettes per day. During the screening, height and weight measurements were taken to ascertain BMI. Only men with a BMI of between 18.5 and 30 kg/m^2 were recruited then stratified into two age-matched groups defined by BMI as either below (normal weight: lean) or above (OW) a BMI of 25 kg/m^2, for analysis, respectively.

2.2 | Imaging sessions

For both of the imaging sessions, participants were instructed to follow an overnight (~12-hour) fast, with their last meal to be consumed no later than 10:00 PM the night before the study visit. Participants abstained from alcohol consumption the night before and caffeine consumption each morning. Shortly after their arrival, participants provided a blood sample via venepuncture from the cubital vein (referred herein as predose) and a second sample after the MRI protocol (referred herein as postscan), ~2.5 hours apart. Blood samples were analysed for plasma glucose, serum insulin and serum C-peptide to assess the effect of IN-INS administration on peripheral concentrations.

2.3 | Intranasal administration

Thirty minutes prior to functional image acquisition, participants received either 160 IU insulin (Humulin, 500 IU/mL, Eli Lily, USA) or saline solution 0.9% w/v (placebo) using a commercial spray device that had been tested and characterized with the used dosage.[18] Administration was timed so that data acquisition coincided with peak insulin concentrations in the central nervous system, in accordance with the pharmacokinetics of IN-INS previously reported.[5]

Administration was performed using a commercial pump with suitable spray characteristics for nose to brain delivery of insulin solution, using an identical dose to this report.[18] Participants had been familiarized with the mechanics of intranasal application at the screening session and self-administered the dose under instruction from the lead investigator. Participants took a total of four spray doses of 40 IU in succession, alternating between both nostrils and leaving 1 minute after each spray to allow time for dissipation and to avoid the solution running out of their nostrils. Full administration details can be found in Wingrove et al.[18] Blinding and randomization were performed by the pharmacy team who prepared the study nasal pump with insulin/placebo solution on the morning of each study day. Neither the participant nor the investigator was aware of what solution was in the nasal pump. Only after the final participant had completed was unblinding performed for the analysis.

2.4 | Blood analysis

Venous blood samples were spun in a centrifuge (10 minutes at 1000 rpm). Plasma and serum were extracted into aliquots and stored at −20°C. Biochemical analysis was performed using routine assays to ascertain serum insulin and C-peptide (Siemens Healthcare Centaur Assays) and plasma glucose (Siemens Healthcare AVIDA) concentrations, respectively. Baseline measures (predose) of insulin sensitivity for each participant were calculated using the homeostatic model assessment of insulin resistance (HOMA-IR) 2 model.[19] HOMA-IR can be calculated using plasma glucose and serum insulin concentrations or plasma glucose with serum C-peptide concentrations. For this study, the latter was implemented using the online, publicly available HOMA-IR 2 calculator v. 2.2.3 (https://www.dtu.ox.ac.uk/homacalculator/). Average HOMA-IR scores across visits were calculated and compared between groups (unpaired t-test).

The change (Δ) in concentration between predose and postdose collection periods was calculated for each metabolite. These Δ values, for each metabolite, were entered into a mixed effects analysis of variance (ANOVA) model to interrogate the main effects of 'Treatment', 'Group' and any interaction effects. Significance thresholds for main effects and interaction effects were set to P less than .017 (0.05/3) to correct for multiple comparisons. Main effects were interrogated with planned comparison t-tests as a post hoc analysis (within-group or within-treatment), and interaction effects with Tukey tests. Planned comparison test significance thresholds were set to P less than .025 to account for the two tests.

2.5 | Hunger scores

Hunger scores were assessed in the scanner immediately after perfusion image acquisition using a visual analogue scale ('How hungry do you feel right now?': '0' = not hungry at all to '100' = very hungry). Similar to the blood analysis above, these scores were run through a factorial model (ANOVA) to look for treatment, group and interaction effects. Significance thresholds were set to P greater than .05. Post hoc analysis was the same as implemented for blood analysis.

2.6 | Image acquisition

Scanning was conducted using a 3 T MR750 GE Discovery Scanner (General Electric, Waukesha, WI, USA) with a 32-channel receive-only head coil. T1-weighted images were acquired using a 3D magnetization prepared rapid acquisition gradient recalled echo sequence with the following parameter: slice thickness (Δz) = 1.2 mm, slices = 196, repetition time (TR) = 7.312 ms, echo time (TE) = 3.016 ms, inversion time = 400 ms, flip angle = 11°, matrix size = 256 × 256 with field of view = 27 cm. The acquisition time was 5 minutes 37 seconds.

Following structural image acquisition, whole-brain CBF data were collected using a 3D pseudo-continuous ASL (pCASL) sequence acquired with a fast spin echo stack of spiral readout. The following parameters were used for the readout: 10 spiral arms, 600 points per arm, leading to an equivalent in-plane resolution of 2.94 × 2.94 mm^2, slice thickness = 3 mm, 54 slices and TE/TR = 11.8/7325 ms. For the perfusion labelling module, the following parameters were used: label duration = 3500 ms, postlabel delay (PLD) = 2025 ms, four background suppression pulses and two pairs of 'control and labelled' images. The total acquisition time was 5 minutes and 37 seconds. A 3D proton density (PD) image was acquired as part of the same image series, using identical readout parameters. This permitted rCBF quantification in standard physiological units, following the methodology recently recommended by the ASL community.[20] Participants were instructed to look at a fixation cross displayed to them via a projector screen.

2.7 | Perfusion image processing and analysis

Quantitative CBF maps were computed from perfusion-weighted (PW) and PD images using the single compartment model and online GE scanner software, according to the formula for single PLD pCASL data from the ASL consensus paper.[20]

2.8 | Image registration and processing

Image processing was conducted using a bespoke pipeline consisting of a mixture of processing software. First, 3D T1-weighted images were combined to create a group template (*templatecreate*, advanced normalization tools software[21,22] [ANTs]), which was registered to

standardized Montreal Neurological Institute (MNI) space. All individual subject transformation and warp matrices from these steps were saved for later application.

PD images, which are in perfect registration, and boast higher tissue contrast, to both CBF maps and PW images, were co-registered to subject-specific T1-weighted images (epi-reg, FMRIB Software Library, v. 3.20, University of Oxford, UK; http://www.fmrib.ox.ac.uk/fsl). Subject CBF maps were normalized to MNI space by applying the saved transformation matrices (*antsapplytransforms*, ANTs) and smoothed using a full width at half maximum Gaussian kernel of $6 \times 6 \times 6$ mm^3 with statistical parametric mapping (SPM) software (SPM-12, Wellcome Trust Centre for Neuroimaging, University College London, UK; www.fil.ion.ucl.ac.uk/spm). This smoothing kernel, of approximately twice the acquired spatial resolution, was implemented in reference to recommendations for group statistical inferences with functional blood oxygen level-dependent (BOLD) data.[23]

2.9 | CBF image analysis

CBF data were analysed at the group level using SPM-12. To measure treatment effects and interactions, CBF maps were entered into a random effects, second-level, voxel-wise, repeated measures factorial model analysis with three factors of 'subject', 'treatment' (IN-PLA, IN-INS) and 'group' (lean, OW) (known in SPM as a 'flexible factorial model'). The inclusion of the subject factor as an explanatory variable to encode between-subject variability helps to model a significant amount of otherwise unexplained variance within the data and has been shown to increase detection sensitivity within pharmacological fMRI experiments.[16] Mean grey matter (GM) CBF values, calculated from a liberal GM mask, were added to this model as a covariate to account for within-subject between-session variability in global perfusion. Analysis was constrained to those voxels within the GM by applying an implicit GM mask. Contrast T-maps for the main effects of IN-PLA versus IN-INS were created as well as interaction effect contrasts, 'Treatment' x 'Group'. Voxel-wise whole-brain analysis results were created from a cluster-forming threshold of P less than .001. Significant clusters were determined based on correction for multiple comparisons computed from 'cluster extent' statistics[24] using a family-wise error threshold (FWE) of P less than .05.

In response to a significant treatment or interaction effect ($P < .05$, FWE), second-level, whole-brain, post hoc paired t-tests were created for each group for the appropriate treatment directionally, using the same cluster-forming and significance criteria described above.

Whole-brain group effects were assessed using separate within-treatment, two-sample t-tests with the same statistical criteria mentioned above and with global GM added as a covariate.

Furthermore, we sought to explore changes in rCBF through employing a region of interest (ROI) analysis with four anatomical, a priori-defined ROIs. Bilateral anatomical regions were selected based on high insulin receptor density profiles and previous publications showing insulin-induced responses and potential functions in food intake and behaviour.[6,25] Insulin receptor-dense regions were defined as the hippocampus and amygdala[25] and the ROIs based on previous IN-INS literature were the insula and the putamen.[6] All subcortical bilateral ROIs were defined in SPM-12 using the fMRI tool of the Wake Forest University School of Medicine (known as the 'WFU pick atlas'; https://school.wakehealth.edu/Research/Labs/Radiology-Informatics-and-Image-Processing-Laboratory/Software-Development#PickAtlas) for implementation of the automated anatomical labelling atlas.

Median CBF values for each individual subject and ROI were extracted (3dmaskave, Analysis of Functional Neuro Images) and entered into a repeated measure ANOVA (rm-ANOVA) statistical model. To correct for multiple comparisons, a Bonferroni significance threshold was set to P less than .0125 (0.05/no. of ROIs) for these tests. Following a significant main effect of treatment or group, planned comparison t-tests were conducted to interrogate treatment (paired) or group (unpaired) related changes. These t-tests were referenced as planned comparisons and formed a post hoc analysis, as opposed to testing all possible combinations. Significance for these planned comparisons was set to P less than .025. Finally, we investigated if global GM CBF was affected by IN-INS or differed between groups to ascertain whether significant effects from ROI analysis may be attributed to global changes and not regional changes in CBF. To this end, GM CBF was extracted from the GM mask employed during the whole-brain analysis and run through an rm-ANOVA model, just like the ROIs above.

2.10 | Presentation of statistical results

Summary data are presented as mean ± standard deviation (SD), tabulated and in graphical formats. Blood and ROI statistical analyses were conducted using R statistical analysis software (*Rstudio* v. 1.1453; Boston, MA, USA; http://www.rstudio.org/).

3 | RESULTS

Thirty participants completed the study. Of these, three were excluded for violation of the fasting study protocol as judged by serum insulin levels (>50 mIU/L); another participant was excluded after presenting with an extreme lack of sleep prior to one of the study visits. The remaining 26 subjects were divided into two groups: normal weight (lean, n = 12, BMI = 22.40 ± 1.89 kg/m^2) and OW (n = 14, BMI = 27.76 ± 1.92 kg/m^2). The two groups were matched for age (27.00 ± 5.44 and 24.76 ± 4.30 years, respectively; P = .30, unpaired t-test) (demographic data are presented in Table 1).

3.1 | Blood analysis

HOMA-IR values did not significantly differ between groups (Table 1). Blood metabolite concentration changes (Δ = [postscan] − [predose]) were interrogated across BMI groups and treatments using an rm-ANOVA model. Blood analysis was performed on data from only

TABLE 1 Table of demographics

	All (n = 26)	Lean (n = 12)	OW (n = 14)	*P*-value
Age (years)	25.9 ± 4.8	27.0 ± 5.4	25.0 ± 4.3	.31
BMI (m/kg^2)	25.1 ± 3.1	22.4 ± 1.9	27.5 ± 1.7	<.001
HOMA-IR	0.97 ± 0.32	0.87 ± 0.22	1.04 ± 0.37	.17

Abbreviations: BMI, body mass index; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; OW, overweight.
Note: Groups were compared using unpaired t-tests. Age and HOMA-IR did not significantly differ between groups.

TABLE 2 Tabulated results of the metabolite and hormone analysis and hunger scores

	Lean (n = 10)		Overweight (n = 13)		*P*-values		
	IN-PLA	IN-INS	IN-PLA	IN-INS	Treatment	Group	Treatment × group interaction
Δ plasma glucose (mmol/L)	−0.03 ± 0.06	−0.2 ± 0.06	−0.03 ± 0.07	−0.12 ± 0.44	.15	.61	.57
Δ serum insulin (mIU/L)	1.23 ± 1.75	3.23 ± 8.25	−0.29 ± 1.57	6.85 ± 3.68	.31	.82	.57
Δ serum C-peptide (pmol/L)	**−46.83 ± 14.75**	**−124.67 ± 27.90**	−56.69 ± 20.60	−132.53 ± 52.61	**.025***	.79	.97
Hunger (0-100)	62.58 ± 6.19	59.91 ± 5.22	62.00 ± 5.10	64.50 ± 4.70	.98	.71	.63

Note: Changes in C-peptide were significantly different between treatments, a greater suppression of C-peptide following IN-INS compared with that of IN-PLA (ANOVA). Post hoc within-group analysis (paired t-test) showed that this treatment effect was significant only in the lean group but not OW (highlighted in bold). For blood analysis whole group n = 25, lean n = 12, OW = 13.
*P < .017, data are presented as mean ± standard error. Hunger scores recorded show no treatment, group or interaction effects.

25 participants because samples were missing for one participant. Changes in plasma glucose and serum insulin did not reveal any significant group, treatment or interaction effects (Table 2).

Analysis of Δ serum C-peptide concentration revealed a main treatment effect ($F_{[1,46]}$ = 5.39, P = .025, rm-ANOVA). Planned comparison t-tests showed a significant treatment effect in the lean group (t[11] = 2.90, P = .015, paired t-test) but not in the OW group (t[13] = 1.74, P = .19, paired t-test). The significant effect in the lean group indicated that following IN-INS the decrease in serum C-peptide concentration was significantly greater than following IN-PLA administration (Table 2).

3.2 | Hunger score analysis

Summary hunger scores for each group and each treatment are displayed in Table 2. Hunger scores were comparable across treatment and groups and no significant effects differences were identified or interaction effects.

3.3 | Whole-brain analysis

Whole-brain, two-sample tests (within treatment) did not provide any significant clusters for either contrast (lean > OW or lean < OW) following IN-PLA or IN-INS. A flexible factorial ANOVA model was employed at the second level to identify clusters of significant change in rCBF associated with the main effects of IN-INS in both the lean and OW groups combined, and to search for any interaction effects. Main effects of treatment (INS vs. PLA) provided no significant clusters when corrected for multiple comparison (FWE, P < .05 at the cluster level) in either direction.

Two interaction contrasts were tested to look for differential treatment responses between lean and OW groups. For the interaction contrast, 'lean (INS > PLA) > OW (INS > PLA)', a significant, large cluster (726 voxels) situated within the right fusiform gyrus (P = .001, FWE-corrected) (MNI co-ordinates: x = 54, y = −60, z = 6) was identified.

Post hoc testing of the subcontrasts forming this interaction is described below. The opposite interaction contrast did not provide any significant results.

3.4 | Post hoc whole-brain testing

A whole-brain, voxel-wise paired t-test for the lean INS > PLA contrast did not provide any statistically significant changes in rCBF. On the other hand, a voxel-wise paired t-test for the INS < PLA contrast revealed two clusters of significant rCBF change. The spatial extent of these clusters encompassed the right parahippocampal and fusiform gyrus (t[13] = 7.15, P = .017, 1023 voxels, FWE-corrected, paired t-test) in addition to the left insula/putamen and hippocampus (t[13] = 8.30, P = .001, 623 voxels, FWE-corrected, paired t-test), respectively, a finding that was observed only in the OW group. MNI co-ordinates and t-values are presented in Table 3; statistical maps are presented in Figure 1.

TABLE 3 Anatomical regions, T scores and MNI co-ordinates for the significant clusters seen from whole-brain parametric paired t-test in the OW group

Region	INS < PLA whole-brain results - OW			*P*-value
	Cluster size (voxels)	T-score	MNI co-ordinates	FWE-corrected
Left hippocampus, insula, putamen, parahippocampal gyrus	623	8.30	−34 −4 −4	.0011
Right fusiform and parahippocampal gyrus	1023	7.14	30 −40 −10	<.001

Note: The large significant clusters had peaks that spanned several anatomical regions.

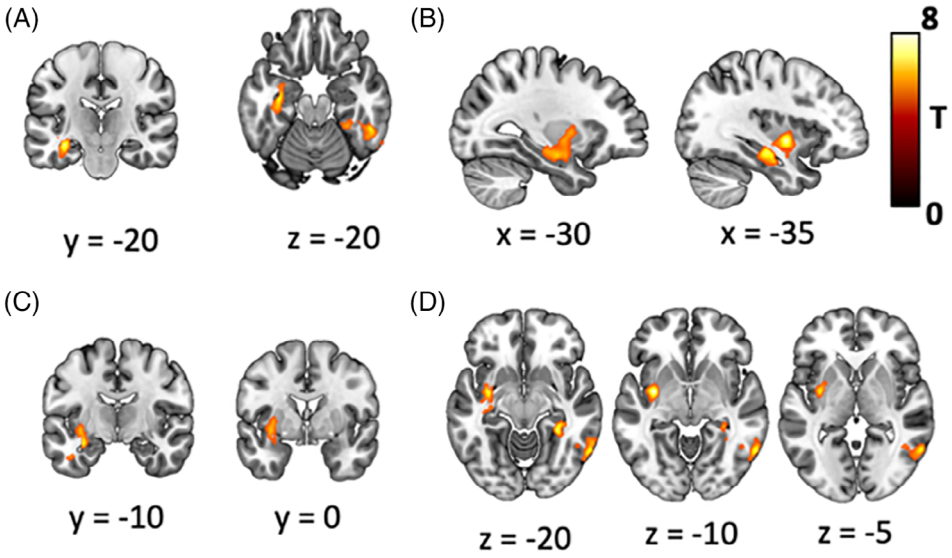

FIGURE 1 Parametric T maps overlaid onto a structural MNI template, illustrating regions of lower IN-INS-related rCBF in OW individuals versus IN-PLA. A, coronal and axial sections displaying the left hippocampal region t-map cluster associated with an IN-INS-related decrease in rCBF and the right parahippocampal gyrus. B, sagittal sections displaying both the left hippocampal and posterior insula-related decreases in rCBF. C, coronal sections showing IN-INS-related rCBF decreases localized to the left insula and putamen regions. D, axial sections highlighting left putamen and posterior insula as well as right parahippocampal and fusiform gyrus decreases in rCBF following IN-INS compared with IN-PLA. Slice co-ordinates are detailed below each image. MNI, Montreal Neurological Institute; OW, overweight

3.5 | ROI analyses

All ROI CBF values are displayed in Table 3. There were no significant treatment, group or interaction effects on global GM CBF. ROI analysis was performed using four previously defined anatomical ROIs. These values were compared across treatment conditions and also across groups to examine rCBF differences through creation of individual ROI rm-ANOVA models.

No interaction effects or main treatment effects were reported from any of the anatomical ROIs tested; however, significant group-related differences in rCBF were seen for the insula ($F_{[1,47]}$ = 10.28, P = .002) (Table 4).

Post hoc tests reported group-related differences following IN-PLA administration only (t[25] = 2.48, P = .020, unpaired t-test) but not following IN-INS (t[25] = 2.01, P = .06, unpaired t-test). The directionality of this change indicated greater rCBF in the OW group compared with the lean group (Figure 2).

4 | DISCUSSION

This study aimed to identify rCBF change in response to IN-INS following administration with an optimally selected nasal pump[18] and to compare these responses between those with a lean and those with an OW, non-obese, body mass. From this crossover study, we identified, from the OW group only, lower rCBF following IN-INS compared with IN-PLA within the left hippocampus, parahippocampal gyrus, insula and putamen, as well as the right parahippocampal and fusiform gyrus. These rCBF decreases were exclusively observed in OW male participants and not in an age- and sex-matched lean/normal weight group. Previous reports of IN-INS administration have suggested that centrally active insulin has no significant direct vasoactive effects within the central nervous system vasculature.[7,15] Furthermore, if IN-INS were to possess vasoactive effects, this would almost certainly lead to effects on global CBF; however, from our data global GM CBF did not differ significantly between treatments or groups. Therefore,

TABLE 4 Mean extracted rCBF values calculated from the ROIs tested for each group and treatment arm

	Regional CBF mL/100 g/min						
	Lean		OW		*P*-value		
	PLA	INS	PLA	INS	Group	Treatment	Group × treatment
Amygdala	43.3 ± 6.4	42.0 ± 5.9	45.9 ± 6.1	41.6 ± 6.9	.55	.11	.40
Hippocampus	42.8 ± 4.9	42.2 ± 4.5	45.8 ± 4.5	43.6 ± 6.0	.13	.27	.56
Putamen	43.2 ± 5.0	43.5 ± 5.5	47.3 ± 6.5	44.5 ± 6.3	.13	.40	.34
Insula	56.7 ± 6.8	57.6 ± 7.7	63.7 ± 7.6	63.0 ± 5.5	**.002****	.98	.66
Grey matter	56.3 ± 7.9	56.5 ± 6.3	59.4 ± 6.0	60.1 ± 4.8	.06	.79	.88

Note: ROI analysis consisted of a group × treatment ANOVA model for each region with HOMA-IR as an added covariate. *P*-values displayed for main group, treatment and group × treatment interaction effects. The insula showed a significant effect of group on rCBF.
*$P < .05$.
**$P < .01$; lean n = 12, OW n = 14.

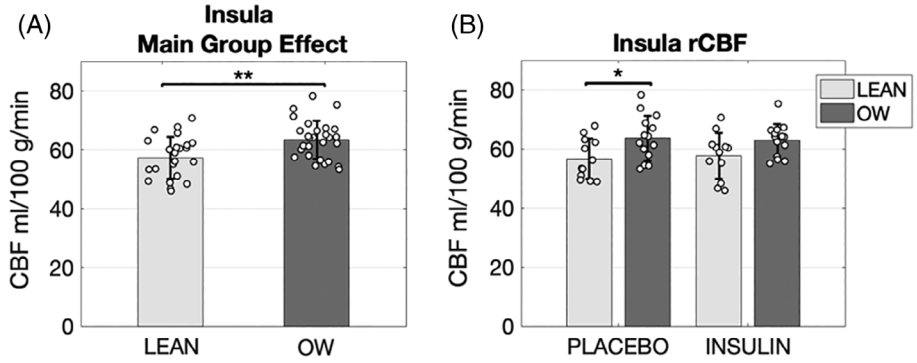

FIGURE 2 rCBF measures extracted from the insula. A, ROI analysis revealed a significant main effect of group where the OW group displayed greater rCBF than the lean group. B, post hoc testing showed that under IN-PLA conditions this group effect is significant but this difference significance under IN-INS conditions. Group average median rCBF values plotted ± SD, with individual subject values (white dots) transposed to show rCBF variability. *$P < .025$; **$P < .01$; lean, n = 12; OW, n = 14; OW, overweight

we have decided that the perfusion differences discussed here can be interpreted to indicate changes in regional neuronal activity.

From an anatomical perspective, the treatment-related changes in rCBF observed from the whole-brain analyses accord with the findings of previous investigations of the effects of centrally acting insulin and also insulin receptor distribution in the brain.[6,7,14,15,26,27] As seen from the results presented in this study there is a difference in observed effects on rCBF between normal weight and OW individuals, and this will be discussed.

Whole-brain statistical parametric maps in OW group analysis displayed lower rCBF following IN-INS versus IN-PLA in two large clusters that spanned regions of the limbic anatomy. Visualization of these maps shows that the clusters encompass the left hippocampus and regions of both the left and right parahippocampal gyrus. This is the first study, to our knowledge, that has reported rCBF changes within the hippocampal formation following IN-INS administration, despite the hippocampus occupying a high density of insulin receptors.[28] Individuals with conditions such as Alzheimer's disease (AD) and T2D display forms of central insulin resistance associated with cognitive and memory impairments, and this has highlighted a possible role of central insulin action in cognitive function and hippocampal physiology.[29–32] Electrophysiology studies in cultivated rat hippocampal slices show that an acute increase in insulin concentration induces recruitment of GABAergic receptors to CA1 synaptic formations.[33] Moreover, insulin signalling within the hippocampus has been shown to hyperpolarize neurons, reducing the neuronal firing rate through interactions with potassium channels.[34,35] Increased GABA-related activity and reduced firing rate within the hippocampus could arguably be sufficient to produce decreases in rCBF, as shown in the current study, and suggests a biological mechanism that underpins the blood flow results presented. The authors would like to highlight the novelty of the results presented and reiterate that despite the preclinical data discussed, this is the first time that IN-INS has shown effects on hippocampal CBF in humans. These data are encouraging as they may provide mechanistic support for the beneficial cognitive and memory effects of intranasal insulin delivery in Alzheimer's disease (AD) and mild cognitive impairment (MCI) clinical trials.[31]

In addition, whole-brain analysis in the OW group showed a large cluster of significant rCBF change at the boundary between the left putamen and the left insula. The putamen, a prominent region within

the limbic system and reward circuitry, has been shown previously to exhibit increases in rCBF following nasal administration of 40 IU insulin in participants of normal weight.[6] The results reported in our study are comparable with regionality but differ in the directionality of rCBF change. This difference could be a result of the higher dose that was implemented in this study (160 IU) or possibly highlights a differential response to IN-INS in this OW group compared with a normal weight group, despite no significant changes being observed in the normal weight group from our study. The higher dosage implemented in this study was decided based on a previous paper by Kullmann et al. that examined dose-dependent effects of IN-INS on resting state activity and CBF.[27] The authors showed and summarized that acute quantification of regional insulin effects with fMRI requires higher doses (160 IU) and that there was a prominent dose-dependent effect with the strongest effects identified from 160 IU dosages.[27] Using this summary and recommendation we opted to implement a 160 IU dose in this study to try and achieve a pronounced fMRI-CBF effect.

The insula is known for its role as a hub for integration of visceral stimuli such as taste and odour, commonly referred to as the primary gustatory cortex,[36] and boasts a high density of insulin receptors.[37] As central insulin activity has shown anorexigenic effects,[38] we looked at hunger levels. There was no change in hunger scores as a result of IN-INS administration or BMI group. The subjects underwent a 12-hour overnight fast, and from our scores showed moderate levels of hunger for both treatment visits. Hunger is often prompted by food-related cues, and even in studies where food intake has been reduced by acute IN-INS (160 IU), hunger scores were not affected.[38] Thus, here hunger ratings were, and perhaps are, of little value for understanding the actual effects on predicted food intake. A previously reported study using a 40 IU IN-INS protocol in lean individuals showed rCBF increases in the bilateral insula.[6] A differential dose-dependent effect of IN-INS[27] within the hypothalamus was identified using an ROI-based approach. The dose-dependent effects showed that higher doses of IN-INS (160 IU) produced prominent decreases in hypothalamic rCBF versus placebo and the lower, 40 IU, dose.[27] In addition, 40 IU administration had no impact on any of the imaging-related measures (CBF or fractional amplitude of low frequency fluctuations). These slightly contrasting effects of 40 and 160 IU suggest a dose-dependent effect of IN-INS that may explain why our findings differ from those of Schilling et al.[6] Of note, Schilling et al. used a short-acting insulin (Actrapid)[6] that was different to the Humulin-U500 used in the current study, and which is longer acting. The authors of the dose-dependent IN-INS study (Kullman et al)[27] did not report what type of insulin (short or long acting for example) they used.

In this work we showed IN-INS-related decreases, compared with IN-PLA, in the right fusiform gyrus for the OW group. Much of the knowledge surrounding this region is drawn from task-based datasets. The fusiform gyrus, along with the hippocampus, has previously been shown to be modulated following IN-INS administration, within a food cue paradigm.[39] The directionality of the treatment effects supports our data but it should be noted that these observations were made in lean individuals while engaged in a visually stimulating task. The fusiform gyrus has been termed an 'insulin-sensitive' brain region from several reviews,[40–42] for which the data reported here provide further support.

Despite OW group treatment effects seen at the whole-brain level, ROI analysis failed to provide any treatment-related differences in rCBF. An explanation for this could be that the a priori regions were bilateral and the results from the whole-brain analysis revealed lateralized IN-INS effects.

The stratification of individuals using BMI allowed the study of individuals in a metabolic state between normal weight and obesity, OW, who could be at risk of developing obesity along with its associated complications.[43] These two groups did not differ in age or peripheral insulin sensitivity. Studying this group is important for establishing possible markers and differences in brain function and/or central insulin signalling that may be associated with this otherwise healthy population. The authors note that by comparing two groups that do not dramatically differ in BMI there is a potential reduction in contrast between groups. In light of this we find that the group-related effects from the ROI analysis are particularly relevant and of interest. No group effects were seen from the whole-brain analysis and could be attributed to a loss of statistical power when identifying peaks across the whole brain in comparison with a more refined ROI approach. Significant group-related differences were identified from the ROI analysis in the insula. These data showed that OW individuals had greater rCBF than normal weight comparators under IN-PLA, which was not observed following IN-INS administration. The observed differences across groups following IN-PLA administration does not, however, describe a modulatory effect of IN-INS. Within each group there was a small change in CBF following IN-INS versus IN-PLA, but what is interesting is that the directionality of these small changes is in accord with the whole-brain interaction effect (i.e. IN-INS-related increase in normal weight and decrease in OW). While these mean treatment changes are small and insignificant, the directionality is sufficient to highlight the statistically significant group effect for IN-PLA conditions and not IN-INS. These differences highlight a regional difference between normal and OW subjects under fasting conditions. Data from fasting resting state fMRI-BOLD, assessing the fractional amplitude of low frequency fluctuations, support insula overactivity with regards to body mass in individuals with obesity compared with normal weight.[44] Furthermore, task-based fMRI research has highlighted the insula as a region involved in the processing of high calorie visual food cues, with increases in insula BOLD activity in individuals with obesity compared with normal weight when viewing high calorie cues.[45] The difference observed here may be relevant for future weight gain and warrants further observational, longitudinal-based study.

Blood metabolite analysis showed no significant treatment effects or group differences in terms of glycaemia or insulin concentration, further supporting the safety of this administration method. We did, however, observe a decrease in C-peptide concentrations over time, a trend seen in the lean group following IN-INS treatment. Remaining fasted for the duration of the study protocol would not require significant pancreatic insulin release and the rate of insulin and C-peptide

release would be below that of degradation during this time,[46] which would explain decreases in C-peptide following IN-PLA. The significant decrease after IN-INS administration in the lean group is compatible with either a systemic spill-over of exogenous insulin that, while not statistically significant, was able to significantly inhibit endogenous insulin secretion, or a centrally regulated negative feedback pancreatic system, as has been suggested previously.[47,48] It is notable that this potential spill-over effect had minimal effects on glycaemia (an average decrease of 0.16 mmol/L) and that significant C-peptide suppression did not occur in the OW group.[48] C-peptide levels did, however, decrease in the OW group under IN-INS but the variability, which was greater than in the lean group, may explain why this was not significant. In addition, insulin resistance in the OW group (although not statistically significant) was slightly higher versus lean and could arguably be a reason why this effect is not seen.

In addition to the dose-dependent effects presented by Kullmann et al., there appear to be differential regional effects on resting state brain activity, as illustrated by IN-INS-related increases in the prefrontal cortex and amygdala, and decreases in the caudate and hypothalamus.[27] Taking all these results into consideration, this makes interpretation of our results, which differ in dosage, cohort and type of insulin, somewhat difficult to situate within the published literature. Of note, there are methodological differences in the literature, not just concerning the aforementioned points but also the implementation of different types of pumps (powder- and solution-based), which ultimately lead to different administration profiles and subsequent central effects.[18]

The results gathered from this examination of the effects of IN-INS administration on cerebral perfusion indicate that insulin is centrally active in this group of OW individuals. We cannot exclude that the lack of average effect in the lean group is possibly attributable to an insufficiently large sample required for the underlying effect size; however, we would prefer to posit a more biological explanation. IN-INS administration may best represent, physiologically, the insulin concentrations that may be seen in the postprandial state following a mixed meal, albeit without the changes in carbohydrate, protein, fat, ghrelin and the plethora of orexigenic gustatory hormones. Regional CBF responses in the insula, striatum (putamen and caudate) and also the hippocampus and fusiform gyrus have been shown to be significantly reduced in response to glucose and fructose ingestion, respectively, in men and women of normal weight.[49] Under this framework and given the lack of effect in the normal weight group, we could suggest that gustatory innervation and/or associated changes in postprandial hormone profiles are necessary for insulin signalling effects on rCBF and that resting neural activity is unaltered by IN-INS in the fasted state. On the other hand, the OW group may, however, have higher resting rCBF in the regions seen from this analysis as a result of factors, not measured, which may lead to insulin-induced reduction in rCBF, similar to that seen under postprandial conditions.[49] This work was performed exclusively in male participants. Previous work has shown the effects of central insulin in men and women to occupy a differential response,[38] and therefore to enhance the power in this work we only recruited males and would be interested in conducting similar research in female-only or mixed cohorts. We report for the first time IN-INS-related changes (vs. IN-PLA) of rCBF in the hippocampus and fusiform gyrus. We conclude that this study showed insulin-induced changes within key regions of the brain involved in gustation, memory and reward in OW healthy male individuals and that under placebo conditions, differences in gustatory rCBF were observed between age-matched OW and normal weight healthy individuals. The differences observed in this study require further interrogation, perhaps through longitudinal-based research, to understand the importance and impact these effects may have. Finally, understanding the effects of central insulin in individuals with a genetic predisposition to accumulating excess fat mass, and not just an OW phenotype—such as those carrying the fat mass and obesity-associated gene risk variant[50]—would help gain more clarity on the central effects of insulin in those more at risk of developing obesity.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

JOW recruited all the participants for the study and carried out all elements of the data collection, data analysis and manuscript preparation. FOZ and SAA were the two main editors of the manuscript and provided guidance to JOW during the interpretation phases. OO aided and provided support with functional image analysis as well as edits and insightful comments to the manuscript. BF and MS provided pharmacy support and obtained reagents and insulin preparations for this study.

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DATA AVAILABILITY STATEMENT

Date will be made available upon reasonable request

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REFERENCES

1. Barker C. *Obesity statistics: Briefing paper*. Vol. number 3336, 2018. London, UK: House of Commons Library.
2. Taylor R. Insulin resistance and type 2 diabetes. *Diabetes*. 2012;61(4):778-779.

3. Kullmann S, Heni M, Fritsche A, Preissl H. Insulin action in the human brain: evidence from neuroimaging studies. *J Neuroendocrinol*. 2015; 27(6):419-423.
4. Henkin RI. Inhaled insulin-intrapulmonary, intranasal, and other routes of administration: mechanisms of action. *Nutrition*. 2010;26(1):33-39.
5. Born J, Lange T, Kern W, McGregor GP, Bickel U, Fehm HL. Sniffing neuropeptides: a transnasal approach to the human brain. *Nat Neurosci*. 2002;5(516):514-516.
6. Schilling TM, Ferreira de Sá DS, Westerhausen R, et al. Intranasal insulin increases regional cerebral blood flow in the insular cortex in men independently of cortisol manipulation. *Hum Brain Mapp*. 2014; 35(5):1944-1956.
7. Grichisch Y, Çavuşoğlu M, Preissl H, et al. Differential effects of intranasal insulin and caffeine on cerebral blood flow. *Hum Brain Mapp*. 2012;33(2):280-287.
8. Harris JJ, Reynell C, Attwell D. The physiology of developmental changes in BOLD functional imaging signals. *Dev Cogn Neurosci*. 2011;1(3):199-216.
9. Nuttall FQ. Body mass index: obesity, BMI, and health: a critical review. *Nutr Today*. 2015;50(3):117-128.
10. Ganz ML, Wintfeld N, Li Q, Alas V, Langer J, Hammer M. The association of body mass index with the risk of type 2 diabetes: a case-control study nested in an electronic health records system in the United States. *Diabetol Metab Syndr*. 2014;6(1):50.
11. Chen Y, Copeland WK, Vedanthan R, et al. Association between body mass index and cardiovascular disease mortality in east Asians and south Asians: pooled analysis of prospective data from the Asia Cohort Consortium. *BMJ*. 2013;347:f5446.
12. Bhaskaran K, dos-Santos-Silva I, Leon DA, Douglas IJ, Smeeth L. Association of BMI with overall and cause-specific mortality: a population-based cohort study of 6 million adults in the UK. *Lancet Diabetes Endocrinol*. 2018;6(12):944-953.
13. Hallschmid M, Benedict C, Schultes B, Born J, Kern W. Obese men respond to cognitive but not to catabolic brain insulin signaling. *Int J Obes*. 2008;32(2):275-282.
14. Kullmann S, Heni M, Veit R, et al. Selective insulin resistance in homeostatic and cognitive control brain areas in overweight and obese adults. *Diabetes Care*. 2015;38(6):1044-1050.
15. Akintola AA, Van Opstal AM, Westendorp RG, Postmus I, Van der Grond J, Van Heemst D. Effect of intranasally administered insulin on cerebral blood flow and perfusion; a randomized experiment in young and older adults. *Aging*. 2017;9(3):790-802.
16. Mehta MA, O'Daly OG. Pharmacological application of fMRI. *Methods Mol Biol*. 2011;711:551-565.
17. Kleinridders A, Ferris HA, Cai W, Kahn CR. Insulin action in brain regulates systemic metabolism and brain function. *Diabetes*. 2014;63(7): 2232-2243.
18. Wingrove J, Swedrowska M, Scherließ R, et al. Characterisation of nasal devices for delivery of insulin to the brain and evaluation in humans using functional magnetic resonance imaging. *J Control Release*. 2019;302:140-147.
19. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985;28(7):412-419.
20. Alsop DC, Detre JA, Golay X, et al. Recommended implementation of arterial spin-labeled perfusion MRI for clinical applications: a consensus of the ISMRM perfusion study group and the European consortium for ASL in dementia. *Magn Reson Med*. 2015;73:102–116.
21. Avants BB, Tustison NJ, Stauffer M, Song G, Wu B, Gee JC. The insight ToolKit image registration framework. *Front Neuroinform*. 2014;8:44.
22. Avants BB, Tustison NJ, Song G, Cook PA, Klein A, Gee JC. A reproducible evaluation of ANTs similarity metric performance in brain image registration. *Neuroimage*. 2011;54(3):2033-2044.
23. Worsley KJ, Friston KJ. Analysis of fMRI time-series revisited—again. *Neuroimage*. 1995;2(3):173-181.
24. Friston KJ, Holmes A, Poline JB, Price CJ, Frith CD. Detecting activations in PET and fMRI: levels of inference and power. *Neuroimage*. 1996;4(3 Pt 1):223-235.
25. Schulingkamp RJ, Pagano TC, Hung D, Raffa RB. Insulin receptors and insulin action in the brain: review and clinical implications. *Neurosci Biobehav Rev*. 2000;24(8):855-872.
26. Kullmann S, Frank S, Heni M, et al. Intranasal insulin modulates intrinsic reward and prefrontal circuitry of the human brain in lean women. *Neuroendocrinology*. 2013;97(2):176-182.
27. Kullmann S, Veit R, Peter A, et al. Dose-dependent effects of intranasal insulin on resting-state brain activity. *J Clin Endocrinol Metab*. 2018;103(1):253-262.
28. Plum L, Schubert M, Brüning JC. The role of insulin receptor signaling in the brain. *Trends Endocrinol Metab*. 2005;16(2):59-65.
29. Ott V, Benedict C, Schultes B, Born J, Hallschmid M. Intranasal administration of insulin to the brain impacts cognitive function and peripheral metabolism. *Diabetes Obes Metab*. 2012;14(3):214-221.
30. Benedict C, Hallschmid M, Hatke A, et al. Intranasal insulin improves memory in humans. *Psychoneuroendocrinology*. 2004;29(10):1326-1334.
31. Craft S, Baker LD, Montine TJ, et al. Intranasal insulin therapy for Alzheimer disease and amnestic mild cognitive impairment: a pilot clinical trial. *Arch Neurol*. 2012;69(1):29-38.
32. Craft S, Peskind E, Schwartz MW, Schellenberg GD, Raskind M, Porte D. Cerebrospinal fluid and plasma insulin levels in Alzheimer's disease: relationship to severity of dementia and apolipoprotein E genotype. *Neurology*. 1998;50(1):164-168.
33. Wan Q, Xiong ZG, Man HY, et al. Recruitment of functional GABA(a) receptors to postsynaptic domains by insulin. *Nature*. 1997;388 (6643):686-690.
34. O'Malley D, Harvey J. MAPK-dependent Actin cytoskeletal reorganization underlies BK channel activation by insulin. *Eur J Neurosci*. 2007;25(3):673-682.
35. O'Malley D, Shanley LJ, Harvey J. Insulin inhibits rat hippocampal neurones via activation of ATP-sensitive K+ and large conductance Ca2+–activated K+ channels. *Neuropharmacology*. 2003;44(7): 855-863.
36. Rolls ET. Brain mechanisms underlying flavour and appetite. *Philos Trans R Soc Lond B Biol Sci*. 2006;361(1471):1123-1136.
37. Werther GA, Hogg A, Oldfield BJ, et al. Localization and characterization of insulin receptors in rat brain and pituitary gland using in vitro autoradiography and computerized densitometry. *Endocrinology*. 1987;121(4):1562-1570.
38. Benedict C, Kern W, Schultes B, Born J, Hallschmid M. Differential sensitivity of men and women to anorexigenic and memory-improving effects of intranasal insulin. *J Clin Endocrinol Metab*. 2008; 93(4):1339-1344.
39. Guthoff M, Grichisch Y, Canova C, et al. Insulin modulates food-related activity in the central nervous system. *J Clin Endocrinol Metab*. 2010;95(2):748-755.
40. Heni M, Kullmann S, Preissl H, Fritsche A, Häring HU. Impaired insulin action in the human brain: causes and metabolic consequences. *Nat Rev Endocrinol*. 2015;11(12):701-711.
41. Häring HU. Novel phenotypes of prediabetes? *Diabetologia*. 2016;59 (9):1806-1818.
42. Kullmann S, Heni M, Hallschmid M, Fritsche A, Preissl H, Häring HU. Brain insulin resistance at the crossroads of metabolic and cognitive disorders in humans. *Physiol Rev*. 2016;96(4):1169-1209.
43. González-Muniesa P, Mártinez-González MA, Hu FB, et al. Obesity. *Nat Rev Dis Primers*. 2017;3:17034.
44. Hogenkamp PS, Zhou W, Dahlberg LS, et al. Higher resting-state activity in reward-related brain circuits in obese versus normal-weight females independent of food intake. *Int J Obes*. 2016;40:1687-1692.

45. Rothemund Y, Preuschhof C, Bohner G, et al. Differential activation of the dorsal striatum by high-calorie visual food stimuli in obese individuals. *Neuroimage*. 2007;37(2):410-421.
46. Matthews DR, Rudenski AS, Burnett MA, Darling P, Turner RC. The half-life of endogenous insulin and C-peptide in man assessed by somatostatin suppression. *Clin Endocrinol*. 1985;23(1):71-79.
47. Schmid V, Kullmann S, Gfrörer W, et al. Safety of intranasal human insulin: a review. *Diabetes Obes Metab*. 2018;20(7):1563-1577.
48. Elahi D, Nagulesparan M, Hershcopf RJ, et al. Feedback inhibition of insulin secretion by insulin: relation to the hyperinsulinemia of obesity. *N Engl J Med*. 1982;306(20):1196-1202.
49. Page KA, Chan O, Arora J, et al. Effects of fructose vs glucose on regional cerebral blood flow in brain regions involved with appetite and reward pathways. *JAMA*. 2013;309(1):63-70.
50. Karra E, O'Daly OG, Choudhury AI, et al. A link between FTO, ghrelin, and impaired brain food-cue responsivity. *J Clin Invest*. 2013;123(8):3539-3551.

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